

THE EFFECT OF CULTURE DENSITY ON SEVERAL OF THE PROPERTIES OF CULTURED CELLS

Brian McCaldin

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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A thesis submitted to the University
of St. Andrews for the degree of
Doctor of Philosophy

by
BRIAN M^CCALDIN

Department of Physiology and
Pharmacology,
University of St. Andrews

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ABSTRACT

A sucrose gradient technique was developed for the preparation of plasma membrane material from SV40 3T3, Py3T3 and HeLa cells. A modification of this method yielded purified nuclei. The plasma membrane material, which was enriched in both 5'-nucleotidase and (Na+K)-ATPase activities, was used to establish some of the properties of the latter in 3T3 and SV40 3T3 cells. Many of these properties were similar in both cell lines.

The effect of conditioning of the growth medium, on the transport of ^{86}Rb into 3T3 and SV40 3T3 cells was investigated. It was shown that conditioning reduced the uptake from growth media and that when the cells were then transferred to Krebs solution, the uptake was apparently increased.

The effect of cell population density on several of the properties of 3T3, SV40 3T3, Py3T3 and HeLa cells was examined. When transformed 3T3 cells were grown in medium containing 10% calf serum, the activities of the cell surface enzymes, (Na+K)-ATPase and 5'-nucleotidase, decreased differently with increasing density. The (Na+K)-ATPase activity decreased sharply as the density exceeded 5×10^4 cells/cm² while the 5'-nucleotidase activity decreased gradually as the density increased. The activity of the latter plateaued so that the overall decrease in the activities of both enzymes was similar. When 3T3 cells were grown under the same conditions, the activities of the cell surface enzymes increased with cell population density. The cell surface enzymes of HeLa cells were shown to decrease with density in a manner similar to those of the transformed 3T3 cells but the decrease in the (Na+K)-ATPase did not exactly follow that of the specific ouabain binding.

The effect of culture density on the intracellular enzymes was shown to depend on the cell type as well as the particular enzyme. In all of the 3T3 cell lines, the acid phosphatase activity was observed to increase with increasing density whereas in HeLa cells this enzyme did not alter. In the transformed 3T3 cell lines, the monoamine oxidase activity did not alter with density and the rotenone insensitive NADH-ferricyanide reductase activity increased but in 3T3 cells, the former increased and the latter decreased. In foetal calf serum, the cell surface enzymes were observed to alter differently with respect to cell population density.

The cell protein tended to decrease as did the cell volume but these measurements were not directly related. The specific Concanavalin A binding also decreased with cell population density but the decreases were not related to the volume changes.

It was concluded that the activity of many of the enzymes of cultured cells is dependent both on the cell population density and the medium in which the cells are grown.

Th 9133

DEDICATION

I would like to dedicate this thesis to the best grey-haired old mother I have ever had, for all her help and support.

CERTIFICATE

I hereby certify that Brian M^CCaldin has spent eleven terms engaged in research work under my direction, and that he has fulfilled the conditions of General Ordinance No. 12 (Resolution of the University Court No. 1, 1967), and that he is qualified to submit the accompanying thesis for the Degree of Doctor of Philosophy.

DECLARATION

I hereby declare that the research reported in this thesis was carried out by me and that the thesis is my own composition. No part of this work has been previously submitted for a higher degree.

The research was conducted in the Department of Physiology and Pharmacology, United College of St. Salvator and St. Leonard, University of St. Andrews, under the direction of Professor J.F. Lamb.

ACADEMIC RECORD

I first matriculated at the University of St. Andrews in October 1970, and graduated with the degree of B.Sc. Hons.(1st Class) in Physiology and Biochemistry in July 1974. I matriculated as a research student in the Department of Physiology, University of St. Andrews in October 1974.

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GENERAL INTRODUCTION

The purpose of this section is to provide a brief historical background to the material to be described later. Many of the topics, mentioned briefly here, will be developed more fully as they arise.

The art of tissue culture was established by the experiments of Harrison (1907) who demonstrated that it was possible to maintain chick embryo tissue for periods of up to 4 weeks in vitro. In 1912 Carrel found that the lifespan of such a culture could be prolonged by feeding with fresh chick embryo extract.

The growth of single isolated cells in culture, necessary for the formation of clones, proved impossible until 1948, when it was demonstrated that single cells in culture would proliferate if the cells were in contact with very small volumes of growth medium (Sandford et al 1948).

In 1954, Sandford et al concluded that after a prolonged period of growth in vitro, normal animal cells came to resemble those taken from neoplastic tissue. In the same year Abercrombie and Heaysman (1954) observed a reduction in movement of cells as two chick embryo heart explants grew towards one another. This process, 'contact inhibition of movement', was generally associated with a decrease in the rate of division and the term contact inhibition later came to mean inhibition of growth or movement brought about by cell-cell contact. Normal cells during the process of adaptation to growth in vitro lose the facility to become contact inhibited and gain the ability to induce tumour formation in experimental animals.

With mouse cells the process of adaptation to growth in vitro is complete within a few months. During this time the majority of the cells cease to divide and eventually die, so that the new cell line consists of cells which have lost the ability to become 'contact inhibited' and essentially resemble neoplastic cells. Cells of this sort are said to be spontaneously transformed. Todaro and Green (1963) showed that the process of spontaneous transformation was not inevitable and, by reducing cell-cell contact during the process of adaptation, established a cell line (3T3) which remained contact inhibited. The capacity of these cells to induce tumours could not be established since they had been obtained from mice which were not inbred. A subsequent study by Aaronson and Todaro (1968), using inbred mice, showed that the retention of contact inhibition was associated with an inability to produce tumours in experimental animals. As a consequence of these observations, loss of contact inhibition became a method commonly used to identify transformed cells. Such cells in culture produce dense foci and are easily distinguished from normal cells.

The normal cells in culture may be transformed to resemble neoplastic cells by the use of agents known to produce tumours in vivo. Transformation may be achieved by treatment with viruses e.g. Polyoma virus (Stoker 1962) or SV40 virus (Todaro and Green 1964), or with chemical carcinogens (Chen and Hedelberger 1969). The cells resulting from such treatment became 'piled up' and formed dense foci, would grow in low concentrations of serum, and were able to induce tumour formation in vivo.

The properties of transformed and normal cells may be compared as an in vitro model for cancer but it must be borne in mind that 1) normal cells, such as 3T3, were selected for their ability to grow to low saturation density and 2) during growth of these cultures, they gradually come to resemble their transformed counterparts.

The process of contact inhibition is not simply dependent on cell numbers, since the final density of 3T3 cells increases with the serum concentration in the medium (Holley and Kiernan 1968). Neither is it simply a process of exhaustion of essential growth factors, since medium in which cells have grown (conditioned medium) and become contact inhibited will support the growth of non confluent cultures. Furthermore when a contact inhibited culture is 'wounded' by removing cells with a suitable instrument, the cells surrounding the wound will divide and migrate in order to 'heal' it. (Dulbecco 1970).

A note of caution was brought to the investigation of 'contact inhibition' by the observations of Ceccarini and Eagle (1971) who demonstrated that a situation similar to contact inhibition might be induced if the pH of the growth medium was reduced below the optimum level for growth and that growth was reinitiated when the pH was raised.

It has been postulated that many of the consequences of transformation might be mediated by cell proteases. This hypothesis is based on the observations that 3T3 cells may be released from the constraint of contact inhibition by mild protease treatment (Burger 1970; Sefton and Rubin 1970) and that the growth of transformed cells is inhibited in the

presence of protease inhibitors (Schnebli and Burger 1972). The biological activity of these compounds was not affected by immobilization on sepharose or agarose beads, suggesting that the phenomena were surface mediated. The specificity of the various protease inhibitors has, however, not been established and during treatment with these substances the transformed cells are not blocked in G_1 phase of the cell cycle (Schnebli 1974), which is the stage at which contact inhibition of normal cells occurs (Nilausen and Green 1965). Furthermore if cell-cell contact is necessary for contact inhibition, then disruption of these by mild protease treatment might reasonably be expected to reinitiate growth.

Cyclic nucleotides have also been implicated in growth control. When 3T3 cells become quiescent, either due to cell density or depletion of growth factors from the serum, the intracellular level of adenosine 3,'5' monophosphate (cAMP) is raised, and when growth is stimulated the intracellular level of cAMP is reduced (Otten et al 1972). cAMP concentrations are much lower in transformed cells. When these levels are raised by treatment with extracellular dibutrylcAMP or substances known to increase intracellular cAMP, (e.g. theophylline) the transformed cells are arrested in the G_1 phase of the cell cycle (Pardee 1974), attain the flattened morphology characteristic of normal cells and display an increased adhesiveness (Willingham and Carchman 1973).

Emmelot (1977) has suggested that cAMP might function as a second messenger responsible for mediating the effects of cell-cell contact i.e. 'functional' cell-cell contact

would increase intracellular cAMP and bring about a 'decrease of DNA synthesis and finally arrest of cell proliferation'. Transformed cells, by virtue of their increased protease activity (Bosman 1972) which may be contained in the cell surface or leaked into the medium (Schneli and Burger 1972) might make only transient functional contacts and not experience increased intracellular cAMP levels. The role of serum might be accommodated in this model if serum proteins were capable of masking the sites responsible for the formation of functional contacts.

There are several other theories which attempt to explain growth control. These include modulations in monovalent cation, divalent cation and phosphate concentrations, diffusion boundary layers or surface charge effects; in each of these cases it is well nigh impossible to distinguish between cause and effect.

Whatever the mechanism by which transformed cells are released from normal growth control, it is clear that the cell surface becomes altered as a consequence of viral transformation. Often, however, the exact nature of the alteration is in dispute. Such a case involves the Na^+ and K^+ stimulated Mg^{++} dependent ATPase (abbreviated to (Na+K)-ATPase, E.C. .3.6.1.4) which is generally accepted as a component of the sodium pump. Lelievre et al (1971) have demonstrated that the activity of (Na+K)-ATPase and 5'-nucleotidase activities of contact inhibited murine myeloma cells decrease rapidly when the cells come into contact, while the activities of the enzymes in non-contact inhibited cells do not alter. In a subsequent study (Ducouret-Prigent

et al 1975) it was observed that although the activity of the (Na+K)-ATPase in contact inhibited cells was reduced to 5% of control values, the ouabain sensitive potassium influx was essentially unchanged. Banerjee et al (1976) have reported that in murine lymphoblasts (L5178Y) the ouabain sensitive $^{86}\text{Rb}^+$ transport and specific ouabain binding increased eight fold during the "early stationary phase of growth".

Elligsen et al (1974) attempted to correlate (Na+K)-ATPase activity with growth of 3T3 and SV40 transformed cells. On confluence the (Na+K)-ATPase activity of 3T3 cells fell to a level below the limits of detection of a standard assay. Kimelberg and Mayhew (1976) have reported an increase in the levels of ouabain sensitive $^{86}\text{Rb}^+$ influx (4.5 fold) and (Na+K)-ATPase activity (2.5 fold) in 3T3 cells as a consequence of transformation by SV40 virus. These authors also report that at cell densities greater than 4×10^4 cells/cm² the ^{86}Rb influx into 3T3 cells declined although the cells were still growing, SV 3T3 cells on the other hand exhibited a sharp increase in these parameters around 10^5 cells/cm² and thereafter showed a marked decline. Banerjee and Bosmann (1976) also found that the rate of ^{86}Rb transport is significantly increased in virally transformed 3T3 cells. The specific ouabain binding was, however, 'paradoxically reduced'. By way of explanation these authors write 'the paradoxical decrease in [^3H] ouabain binding which may reflect the inadequacy of the rodent cells in measurements of this type and hence be artifactual in nature because of lack of accuracy in the methodology'.

Brown (1976) has reported a decrease in the ^{86}Rb uptake into 3T3 cells on confluence, while, in contrast, the uptake into SV40 and Py 3T3 cells does not alter.

SCOPE OF THE PRESENT INVESTIGATION

The purpose of the present investigation was to observe the effects of cell density on the levels of (Na+K)-ATPase and other enzymes. To preclude the deterioration of enzyme activities during isolation, the activities were measured in the whole cell homogenate. Since there are many other enzymes which will hydrolyse ATP some of the characteristics of the (Na+K)-ATPase were established in preparations of purified plasma membrane before analysis of the enzyme in whole cell homogenates. Since several of the established isolation procedures were attempted, unsuccessfully, a method for the rapid isolation of plasma membrane from cultured cells was established in order to characterise the (Na+K)-ATPase. The effect of 'conditioning' of the medium, during the growth of cultures, on the transport of rubidium was investigated since it was thought that the apparently contradictory reports concerning ^{86}Rb transport and activity of the (Na+K)-ATPase might be a consequence of conditioning. Several of the properties of the cell were examined in relation to cell density in order to establish whether the loss of (Na+K)-ATPase activity was a specific phenomenon or related to a more general and complex series of events.

CHAPTER 1

THE RAPID ISOLATION OF PLASMA MEMBRANE
AND NUCLEAR ENVELOPE FROM CULTURED CELLS

SUMMARY

1. A sucrose gradient method was developed for the isolation of plasma membrane material from SV40 3T3 cells. The effects of homogenisation conditions, divalent cation concentration, composition of the sucrose gradient and sample loading density were examined.
2. Homogenisation was achieved by passing the cell suspension repeatedly through a stainless steel syringe needle of internal diameter 0.41mm. The number of passages for complete disruption depended on the cell type. The number of passages required increased in the following manner SV40 3T3, Py3T3, 3T3, HeLa. The homogenate was adjusted to a known sucrose concentration and layered on gradient I or II (see methods section).
3. The material which banded at each interface was aspirated off and pelleted by centrifugation in order to remove the sucrose.
4. Gradient I was used to determine the effects of alterations in the divalent cation concentration. As this parameter was varied, the distributions of 5'-nucleotidase (E.C. 3.1.3.5), rotenone insensitive NADH-ferricyanide reductase (E.C. 1.6.99.3) and succinate dehydrogenase (E.C. 1.3.99.1) activities were observed to alter. On

the basis of the recovery of these marker enzymes, the optimum plasma membrane isolation was obtained between the 25% and 30% sucrose layers (band II plate I) when the divalent cation composition of isolation medium was 1mM MgCl_2 .

5. The isolation provided by gradient II under these conditions was less effective and the results obtained using Potter homogenisation in conjunction with gradient I proved less reproducible.
6. In the presence of 1mM MgCl_2 no (Na+K)-ATPase (E.C. 3.6.1.4) activity could be detected in the homogenate or any of the gradient fractions. The inclusion of 0.1mM EDTA in the isolation medium enabled the detection of (Na+K)-ATPase activity suggesting that heavy metal ions might be responsible for the inactivation of this enzyme. While the inclusion of 0.1mM EDTA enabled the detection of the latter, the 5'-nucleotidase activity was greatly reduced. The activity of 5'-nucleotidase could, however, be preserved by the addition of CaCl_2 to the isolation buffer.
7. When the isolation buffer consisted of 10mM trisHCl (pH 7.4 at 4°C) 0.2mM MgCl_2 , 0.5mM CaCl_2 and 0.1mM EDTA and the homogenate was layered onto gradient I as a suspension 40% (w/v) with respect to sucrose, the maximum relative specific activities of both (Na+K)-ATPase and 5'-nucleotidase were obtained at the interface between

the 30% and 35% sucrose layers (band III plate I). In this case the relative specific activity of the (Na+K)-ATPase was twice that of the 5'-nucleotidase.

8. Under the above conditions the activities of acid phosphatase (E.C. 3.1.3.2) monoamine oxidase (E.C. 1.4.3.4), rotenone insensitive NADH-ferricyanide reductase (E.C. 1.3.99.1) and succinate ferricyanide reductase were also estimated. The greater proportion of these enzymes were obtained in the position, on the gradient, which would have been predicted from the reported bouyant density of their respective organelles.
9. Nuclear material, prepared under the above conditions was largely contaminated with monoamine oxidase and acid phosphatase activities. However, this contamination was reduced if, prior to loading on the gradient, the homogenate was adjusted to 55% (w/v) with respect to sucrose. If the pellet from gradient I obtained with a loading density of 40%(w/v) sucrose, was resuspended to 55%(w/v) in sucrose and recentrifuged on the above gradient , the contaminants were not removed.
10. Under the conditions optimal for the preparation of nuclear material, the plasma membrane fraction had a high level of contamination by other organelles. Therefore, it was technically not possible to obtain both plasma membrane material and nuclei from the same gradient.

11. Plasma membrane could be prepared from Py3T3 cells by the method developed for SV40 3T3 cells. In this case the purification of (Na+K)-ATPase and 5'-nucleotidase activities in the plasma membrane fraction were comparable to those obtained with SV40 3T3 cells and the relative specific activity of the (Na+K)-ATPase was twice that of the 5'-nucleotidase. The distributions of the other marker enzymes were also similar.
12. Gradients prepared from HeLa cell homogenates contained no monoamine oxidase activity. The purifications of (Na+K)-ATPase and 5'-nucleotidase activities in plasma membrane fractions from HeLa cells were only 6 fold, and the extent of purification of the two enzymes was similar. This situation contrasts with that of the transformed 3T3 cell lines.
13. It was concluded that the developed separation method was rapid, taking less than 3 hrs. in total, and efficient, producing plasma membrane with (Na+K)-ATPase at a specific activity 6-14 fold that of the homogenate. In the hands of the author this method was better than any of the existing methods which were used.

INTRODUCTION

The preparation of a particular cell organelle may conveniently be considered in three stages: 1) Disruption: the process whereby the cells are ruptured prior to 2) Isolation: the removal of the organelle in question from the remainder of the cytoplasmic constituents and 3) quantification: the estimation of the 'purity' of the fraction from the activity or concentration of 'markers' for both the fragment itself and any contaminants which might be present.

Disruption: the first step has a triple aim of (i) disrupting as many of the cells as possible (ii) producing large plasma membrane fragments and (iii) maintaining the structural integrity of the intracellular organelles. The disruption of intracellular organelles is to be avoided since: (i) fragmentation of the mitochondria would result in the liberation of the outer membrane which is of a density comparable to that of the plasma membrane; (ii) the disruption of the nuclei results in the release of basic proteins which induce the non-specific aggregation of negatively charged membrane fragments; and (iii) the lysosomes contain hydrolytic enzymes which on release will degrade other cell components. Since some disruption of organelles is unavoidable the homogenate is usually maintained at the temperature of melting ice (0°C) in order to retard the degradation of organelles by the released hydrolytic enzymes.

The state in which the plasma membrane is released depends on both the method of disruption and the conditions

during homogenisation. The plasma membrane may be released from cells in the form of whole ghosts, large sheets, or open or closed vesicles. The state of the disrupted membrane in turn dictates the most appropriate method of isolation.

Disruption may be achieved by either physical or chemical techniques or by a combination of both. Physical techniques, of which there are a wide variety, rely for the most part on stress distortion of vulnerable membrane regions.

In Potter or Dounce homogenisation, the shearing force experienced by any particle in suspension depends on local viscosity, particle velocity and frequency of collisions with solid surfaces. These parameters bear a complex relationship to bulk viscosity, speed of rotation and clearance of pestle, and cannot be directly controlled.

The process of sonication has several drawbacks. In addition to localised heating effects and the production of free radicals there will be extensive vesiculation of membrane material. Heat and free radicals are likely to produce protein denaturation and the vesicles of the sort produced by sonication are likely to trap cytoplasmic components.

It has been claimed (Wallach 1972) that nitrogen cavitation disruption of the cells by a rapid reduction of pressure in an atmosphere of nitrogen, selectively disrupts the plasma membrane. The original authors (Hunter and Cummerford 1961) however, make only the modest claim, on the basis of S value of deoxyribonucleo-protein complex and P/O ratio of the mitochondria, that nitrogen cavitation is no worse than Dounce homogenisation.

The common chemical methods for disruption include

osmotic stress, proteolytic enzymes, lypolytic enzymes, pH extremes and surfactants. These conditions tend to be non-specific and are likely to result in disruption of internal organelles, with concomitant contamination of the plasma membrane and inactivation of the plasma membrane enzymes. It seems preferable, therefore, to use chemical procedures such as mild hypotonic stress in combination with physical procedures such as gentle mechanical disruption.

Surface hardening techniques permit the isolation of whole cell ghosts from nucleated cells. Isolation of the plasma membrane in such a form is advantageous in view of:

- 1) The possibility of identification of the plasma membrane fraction on a morphological basis precluding the problems associated with the assignment of markers to the cell surface. Organelle markers must, however, be estimated to measure the extent of contamination by other cell components;
- 2) there is the probability that the plasma membrane vesicles may trap cell organelles. Even organelles of a density much greater than the plasma membrane, which would not otherwise be present in plasma membrane preparations, may become contaminants in this way;
- 3) the proposed asymmetry of cultured cell plasma membrane. Cultured cells have three distinct surface regions: a) the cell-cell surface; b) the cell-substratum surface and c) the cell-medium surface. It is possible that these surfaces may have different architectural and enzymic properties. Preferential selection of any one of these components is avoided by the isolation of the cell surface as a whole ghost. Surface hardening may be achieved by at least two methods: 1) the zinc ion method, and 2) the

magnesium ion method (Warren et al 1966). The former involves the use of 1mM ZnCl_2 in hypotonic conditions to produce swelling of the cells which on Dounce homogenisation extrude their nuclei and cytoplasmic constituents. This method has been employed by several authors (Lerner et al 1971; Perdue and Sneider 1970; Brunette and Till 1971; Glick et al 1973; Charalampous et al 1973; Shin and Carraway 1973) although Zn^{++} itself seems to be responsible for the inhibition of several enzymes (Rothfield and Finklestein 1968). The inhibition may be removed by treatment with EDTA but as a consequence some 65% of the membrane protein is lost into solution (Perdue and Sneider 1970). The other method involves the use of 5mM MgCl_2 with 50mM Tris HCl (Warren et al 1966) as a homogenisation buffer. This method permits the isolation as whole ghosts 10-30% of the total cell numbers. The efficacy of divalent cations in the production of membrane aggregates has been reported to be dependent on cell type (Neville 1976) but it is likely that the magnesium ion method would be prone to such difficulties.

Buffering of the homogenate is necessary since extremes of pH have detrimental effects on the activities of several enzymes. Of the many buffering systems in use the two most 'physiological' are bicarbonate and phosphate. However, neither is suitable for this study, since the pH of the former will fluctuate unless a constant ^{partial} pressure of CO_2 is maintained while the latter is unsuitable when it is necessary to estimate the activity of phosphohydrolases by measuring the release of inorganic phosphate. Trizma

base, pH adjusted by the addition of HCl or glycine, is one of the cheapest and most commonly used buffering systems. It may be used effectively in the range 7.1-9.1 at 5°C and, provided that its temperature coefficient is borne in mind, it may be used in isolation procedures.

Separation

Plasma membrane separation may be achieved by chemical techniques or density or differential centrifugation, either alone or in combination. The most widely used chemical technique is the two phase polymer system. In this system the plasma membrane forms an interface, between the two phases, comprising 6-7% of the total cell protein. Brunette and Till (1971) were among the first authors to describe the use of a two phase aqueous polymer system in conjunction with the Zn ion method of Warren (1966). This method has recently become popular with several workers. Lesko et al (1973) compared the product of the two phase polymer method with that obtained from sucrose density gradients and concluded that though the two systems provided similar material, the two phase polymer system yielded 49% more membrane. Israel et al (1973), however, found that after separation by the two phase polymer system, an additional sucrose gradient centrifugation was necessary to reduce contamination to an acceptable level.

The method most frequently used to prepare membrane fragments is centrifugation. The original rotors were of the fixed angle variety and the inhomogeneity of their centrifugal field limited their use to differential pelleting. This method relies on the ability to distinguish between

particles on the basis of differences in the rate at which they will migrate in a centrifugal field (S value). De Duve et al (1955) used differential pelleting to show that cell components which differ in size and density have distinct morphological and biochemical characteristics. The particles released on cell homogenation may be separated into 4 groups which may be further purified by density gradient centrifugation: 1) a crude nuclear fraction; 2) a heavy mitochondrial fraction; 3) a light mitochondrial fraction and 4) a crude microsomal or soluble fraction.

The use of density gradients for the stabilisation of the zones formed during centrifugation was first suggested by Pickles (1943). Anderson (1955) has suggested that there are two distinct types of density centrifugation; differential centrifugation and isopycnic centrifugation. In differential centrifugation the sample is applied as a thin layer to the top of a liquid column through which it then moves under the constraint of a gravitational field. The process is essentially kinetic - the particles move according to their sedimentation coefficients - and may be used for differential pelleting or interrupted at any stage for the removal of the various bands which may have formed. Isopycnic centrifugation is essentially an equilibrium sedimentation. The material to be separated is usually distributed evenly throughout the gradient at the beginning of the run and on application of a gravitational field the particles will move through the gradient towards their equilibrium density.

De Duve et al (1959) have discussed the properties of the ideal gradient material and concluded that apart from the

ability to form a density gradient, it should exert a minimum osmotic effect, produce minimum alterations in viscosity, ionic strength, and pH of the medium and should not be surface active or produce denaturation in the particles to be fractionated. Although sucrose is the most commonly used gradient material the osmotic effects of this substance may be avoided by the use of ficoll or some other large molecular weight compounds.

The physical and biochemical characteristics of the various cell organelles have been well documented and the reader is referred to a table of buoyant density and differential pelleting properties of these structures compiled by Neville (1976) from various sources.

Quantification

If the plasma membrane exists as large sheets its identification may be accomplished on a morphological basis. Contamination, however, must still be estimated by the presence in the plasma membrane fraction of markers for the other cell organelles. Small plasma membrane vesicles on the other hand may be morphologically indistinguishable from microsomal vesicles and must be identified on the basis of their content of markers alone.

Enzymic markers: no enzyme is invariably a marker for the cell surface. 5'nucleotidase (E.C. 3.1.3.5), the enzyme most commonly used in this role, shows only low enrichment in the plasma membrane fractions from some cultured cells (Perdue and Sneider 1970; Shin and Carraway 1973). Its activity has been detected in the Golgi apparatus (Farquhar et al 1974),

and in the E.R. (Widnell 1972). From muscle cells (Schimmel et al 1973), fragments containing (Na+K)-ATPase but deficient in 5' nucleotidase activity have been isolated. 5'-nucleotidase appears to be a good marker for liver bile front but there may be species differences (Lutz 1973). Moreover the 5' nucleotidase activity of fibroblasts is susceptible to alteration during viral transformation (Perdue et al 1971) and cell contact (Lelievre et al 1971).

Adenyl cyclase (E.C. 4.6.1.1) has been proposed as ostensibly the best plasma membrane marker (Solyom and Trams 1972). However, this enzyme may exist in an inhibited or activated form (Riek et al 1970; Constantopoulos and Najjar 1973). In addition, the use of adenyl cyclase as a plasma membrane marker has been challenged on the basis of: 1) the fact that its activity is not enriched in microvillus fractions of rat kidney (Wilfong and Neville 1970); and 2) its reported presence in the sarcoplasmic reticulum (Entman et al 1969) and the Golgi apparatus (McKeel and Jarret 1974).

It is generally accepted that the (Na+K)-ATPase (E.C. 3.6.1.4) is intimately associated with the sodium pump. As a consequence the activity of this enzyme should be located exclusively in the plasma membrane, yet the activity, ~~which~~ according to Glick (1976), is only slightly enriched in some plasma membrane preparations and has been observed in all cell fractions. It is recommended that, where applicable, more than one marker should be employed for each cell fraction. It is also recommended that the total recovery of a marker should be estimated. The former provides corroborative evidence for calculations of purity and a

measurement of the latter is necessary in order to calculate the percentage of a contaminating organelle, since a portion of the enzymic activity might become irreversibly inhibited during isolation.

The nuclei are unlikely to contaminate the plasma membrane fraction due to the large difference in their buoyant densities. The nuclei have a characteristic appearance and contain some 90-95% of the total cell D.N.A. facilitating morphological and chemical identification. The nuclei pose no great problems during the isolation of plasma membrane ~~provided~~, since, as previously stated, their integrity can easily be maintained by the addition, to the isolation buffer, of small quantities (around 1mM) of divalent cations.

The contamination of the plasma membrane fraction by mitochondria should not be problematic nor should the extent of the contamination be difficult to estimate. The intact mitochondria have a buoyant density greater than that of the plasma membrane and possess a characteristic appearance which facilitates morphological identification. Should fragmentation occur, the outer mitochondrial membrane (which has a buoyant density in the same range as the plasma membrane) would become a contaminating factor but the uniform enzymic composition of this organelle would permit its unequivocal identification. The outer membrane contains kynuramine hydroxylase (E.C. 1.4.3.4). The inner mitochondrial membrane which has a buoyant density much greater than that of the plasma membrane contains rotenone sensitive NADH dehydrogenase (E.C. 1.6.99.3) and succinate dehydrogenase (E.C. 1.3.99.1) (see Scottocasa (1976) and Capaldi (1977))

for reviews).

The lysosomal fraction has no typical ultrastructure and may not be identified morphologically. Lysosomes exhibit crypticity since their membranes are impermeable to most of the substrates of their constituent enzymes. The problems associated with obtaining a lysosomal preparation of even moderate purity has made the assignment of markers well nigh impossible. Acid phosphatase (E.C. 3.1.3.2) has a buoyant density peak similar to lysosomes and this enzyme has been used as a lysosomal marker by several authors. (For review see Wattiaux 1977.)

Vesiculated E.R. has a heterogeneous density which overlaps that of the plasma membrane and the separation of these components is difficult to achieve. In liver glucose-6-phosphatase (E.C. 3.1.3.9), G-6-Pase, and a number of electron transport enzymes are found in the E.R.. In other tissues G-6-Pase is present in low amounts and its validity as a marker in these circumstances is questionable. In rabbit aorta smooth muscle and cultured rat embryo fibroblasts, no satisfactory E.R. marker could be found. (For reviews see de Pierre and Dallner (1976) and Gevze et al 1977).

The controversial Golgi apparatus, first observed in the cerebellum of the barn owl, proved very difficult to isolate and its role in the storage and transport of materials (Neutra and Lebond 1966) was elucidated long before any characterised preparation had been produced. The glycosyl transferases were thought to be exclusively associated with the Golgi apparatus (Moore et al 1974), but these are

detectable in nuclear envelope (Agguter, personal communication). Thiamine pyrophosphatase (E.C. 2.5.1.3) which had been thought to act as a marker for the Golgi apparatus has been found in the E.R. and the plasma membrane but it seems unlikely that its presence in the Golgi apparatus (Lo and Marsh 1970) is entirely due to contamination.

Although the peroxisomes have not been extensively characterised they are known to contain catalase (E.C. 1.11.1.6) and flavin oxidase (E.C.) and may play a role in gluconeogenesis (de Duve and Baudhuin 1966). These structures have a buoyant density of 1.20-1.25 g/ml in sucrose gradients and should not contaminate the plasma membrane fraction to any great extent.

Chemical markers: Chemical markers such as sialic acid, fucose, and cholesterol have been shown to be enriched in plasma membrane preparations. The cell surface may contain up to 70% of the total cell sialic acid (Glick et al 1971) but this amount may vary by as much as 25-30% depending on growth conditions and the phase of the cell cycle (Glick 1971). The presence of sialic acid has been reported in the lysosomes (Glick 1971) and Golgi apparatus of liver (Bergman and Dallder 1976).

Fucose is enriched in plasma membrane preparations but its presence in small amounts has been reported in particulate matter throughout the cell (Keshgegian and Glick 1973; Steiner and Melnic 1974).

Cholesterol appears to be a component of plasma membranes (Weinstein et al 1969) but is also present in

lysosomes and constitutes 20-30% of the nuclear envelope lipid.

Membrane Probes

Although membrane probes have been used extensively in the study of the distribution of proteins at the cell surface, to date they have been little used in plasma membrane isolation. There are many reports in the literature of procedures for surface labelling and Maddy (1964) has proposed several criteria which specific labels should fulfil; they should (a) be able to interact with the membrane under physiological conditions of pH and tonicity; (b) be small enough to gain access to the membrane proteins yet remain impermeable and (c) adhere strongly to the membrane to achieve a stable linkage in amounts which can easily be detected.

The mammalian erythrocyte has been the cell most studied using surface membrane probes. The efficacy of the probe is usually determined by comparing the quantity of label attached to the whole ghost with the extent of haemoglobin binding. Maddy (1964) designed one of the first fluorescent labels (4-acetamido-4'-isothiocyano-2,2'-stilbine), SITS, which was thought to react both specifically and covalently with the proteins of the cell surface. It has, however, been shown that this compound reacts noncovalently and reversibly with the cell surface (Cabantchik and Rothstein 1972). A number of other stilbine derivatives have been investigated (Cabantchik and Rothstein 1972) and it appears that DIDS (4,4'-diisothiocyano-stilbine) strongly attaches to the cell

surface without adversely affecting the viability of cultured cells (Juliano and Behar-Bannelier 1975).

PCMBS (p-chloromercuribenzenesulphonate) which has also been used as a membrane probe initially reacts with the cell surface but slowly penetrates the cell (Sutherland et al 1967), forms unstable complexes with surface -SH groups and extracts a large fraction of the protein of erythrocyte ghosts (Godin and Schrier 1972).

Berg (1969) has synthesised DABS (^{35}S diazobenzene sulphonate) which, though it labels the interior of the cell to the extent of only 5%, induces a rapid loss of K^+ , inhibits facilitated glucose transport and acetyl choline esterase activity and is responsible for the degradation of protein (Bender et al 1971).

Brescher (1971) used FMMP (s-formyl-methionyl-sulphone-methyl-phosphate) to study the proteins of the erythrocyte but the labelling was accomplished at pH 10, which might have a detrimental effect on the activity of some enzymes, and 16% of the label was found attached to the haemoglobin indicating that the molecule had penetrated the cell surface.

Rifkin et al (1972) tritiated the surface by the addition of ^3H borohydride to Schiff bases formed by interaction between pyridoxal phosphate and amino groups of the membrane. This procedure did not significantly label the interior of cultured cells (Juliano and Behar-Bannelier 1975) but might be expected to inhibit any enzymes which have an amino group at their active site.

Enzymatic iodination, the use of which has been extensively reviewed by Hubbard and Cohn (1976), would appear

to be the most useful cell surface specific label. This method also has its drawbacks. There is a prevalence of unsaturated fatty acyl residues in the plasma membrane which will be subject to non-enzymic iodination. Such iodination might alter the physical and chemical properties of the membrane. In extreme cases the phase transition temperature may be so radically elevated that access of the label to the interior of the cell will occur. This method has been used to label the cell surface prior to isolation. In these studies ^{125}I was incorporated into the cell surface of intact cells, prior to isolation of the plasma membrane by established methods. The enrichment, assessed as the specific activity of the isolated plasma membrane relative to the whole cell, was shown to be from 6 to 20 fold.

Lectins, proteins obtained from plant material, include wheat germ agglutinin, soya bean agglutinin, phytohaemagglutinin and the more common concanavalin A. These proteins, purified and labelled with ^{125}I or ^3H -acetic anhydride, have been used to study alterations in the cell surface with viral transformation and cell contact (Nicolson and Lacorbiere 1973). Lectins have also been used as markers for the cell surface during isolation and phagocytosis (Oliver et al 1974).

Procedural artifacts

Some of the problems and uncertainties concerning the assignment of enzymes and chemical markers to specific cell fractions may in part be due to procedural artifacts. Enzymic treatment such as trypsinization has been shown to remove 20-30% of the cell surface glycoproteins (Buck et al

1970) and inhibit acetyl cholinesterase (Hall and Kelly 1971). The activity of this enzyme in nerve cell membranes is also dependent on the pH and type of buffer used (Hayden et al 1973). Treatment with EDTA removes cell surface glycoproteins (Glick 1974) and in alkaline conditions solubilises 25% of the membrane protein of liver cells (Gurd et al 1972). In addition to the loss of membrane protein and the *Binding of specific proteins to all age* problems which this poses there may be adsorption of cytoplasmic material onto the plasma membrane *See LBS but not as only on separation* (Kadlubowski 1976). Since material can be lost from and adsorbed onto membranes it is possible that there may be a redistribution of marker enzymes during isolation. Enzyme activation may be envisaged subsequent to release of proteases, glycosidases or lipases from the lysosomes or due to a rearrangement of the membrane proteins themselves. The action of the hydrolytic lysosomal enzymes could activate proenzymes and membrane rearrangement might release them from the constraints of crypticity. The rearrangement of membrane protein is suggested by the differential binding of permeable protein labels such as dansyl chloride (Schmidt-Urlich et al 1973) or acetic anhydride (Carraway et al 1971). These probes should label all of the available binding sites in intact red cells, but labelling is more extensive if lysed cells are used (Bretscher 1973).

In addition to procedural artifacts, much of the quandary over enzymic distribution may be explained in terms of cellular metabolism and membrane flow. The reader is directed to a review on the subject of membrane biogenesis by Malhotra (1976).

The fusion of secretory granules with the cell surface was first observed by Palade (1959). More recently this phenomenon has been studied by following the progression of tritiated sugar residues through the cell. These first appear in the Golgi apparatus and become incorporated into the plasma membrane of HeLa cells during the fusion of small secretory granules (Reith et al 1970). Pinocytosis of extracellular material represents one method whereby the material which constitutes the cell surface might be reclaimed. In this case the membrane fuses with a primary lysosome to form a secondary lysosome in which the membrane constituents may be degraded for reuse.

The above observations have formed the basis of the 'Membrane flow hypothesis' of Morré et al (1971) who propose that 'the biogenesis of certain membranes is accomplished by the physical transfer of membrane material from one cell component to another in the course of formation and normal functioning'. Membrane formed in the E.R. would be incorporated into the Golgi apparatus and hence transported to the plasma membrane during exocytosis. This hypothesis is corroborated to a certain extent by the observations that a) the Golgi apparatus has a composition intermediate to that of the plasma membrane and the E.R. and b) the lipid and protein of the secretory granule are manufactured at the same time as the material for export but have a very much longer half life (Meldolesi and Cova 1972). It seems clear, therefore that the dynamic exchange of material amongst the various intracellular structures makes the isolation of a plasma membrane free from enzymes of other cell organelles

impossible in principle and that at best one may achieve only a steady state representation.

Notwithstanding these difficulties, any study of the enzymes/^{of} the plasma membrane requires the isolation of this organelle in a reasonably pure form. This is especially so in the case of the (Na+K)-ATPase in view of the preponderance in the cell of enzymes which will hydrolyse the same substrate.

MATERIALS

Tissue culture HeLa cells, 3T3 cells, SV40 3T3 cells and Py 3T3 cells were obtained from Flow Laboratories Ltd., (Irvine) and a stock of each cell line, frozen in liquid nitrogen, was maintained in the department. All other materials required for the growth of cells, with the exception of gentamicin, i.e. Dulbecco's modification of Eagle's minimal essential medium (D.M.E.M.), Eagle's Basal medium with Earl's salt solution (B.M.E.), newborn calf serum, ^{foetal} calf serum Trypsin and glutamine were obtained from Flow Laboratories Ltd. (Irvine). Gentamicin was obtained from Rousselle Laboratories Ltd.

General The following items were all purchased from the Sigma Chemical Co. Ltd. :- adenosine 5'triphosphate (ATP, disodium salt, grade II), adenosine 5'diphosphate (ADP, sodium salt, Grade I), adenosine 5'monophosphate (AMP, monosodium salt, type II), β -Nicotinamide adenine dinucleotide, reduced form (NADH, disodium salt, grade III), nicotinamide adenine dinucleotide phosphate, reduced form (NADPH, tetrasodium salt, grade I), Kynuramine hydrobromide, sialic acid (n-acetyl neuraminic acid, type I), Tris base, tetraethylammonium chloride. P-nitrophenyl phosphate (p-NP, disodium salt,) was obtained from Koch-Light Ltd. Ouabain was obtained from Laboratoire Naturelle, Paris. Sodium succinate and rotenone were obtained from British Drug House Ltd. All other reagents were of Analar grade.

METHODS

Cell culture. The cell lines employed in this investigation were initially supplied as monolayer cultures by Flow Laboratories Ltd. (Irvine).

The mouse fibroblast lines, SV40 3T3, Py 3T3 and 3T3 cells were grown in Dulbecco's modification of Eagle's minimal essential medium (Table 1A) supplemented by the addition of calf serum (final concentration 10% v/v) and gentamicin (final concentration 0.04 mg./ml.). HeLa cells were grown in Eagle's basal medium (Table 1.B) containing the same additives.

Suspensions of cells in the appropriate growth medium containing 10% (v/v) dimethylsulphoxide were frozen in liquid nitrogen and stored at -70°C in a Union Carbide LR-40 liquid nitrogen refrigerator. At three monthly intervals cultures were discarded and replaced by cells reactivated from frozen stock.

Cell propagation. All the procedures involved in the propagation of cells were routinely carried out with aseptic precautions in a 'Bassaire' laminar flow cabinet.

The cells in frozen stock were thawed rapidly to prevent the formation of ice crystals and diluted in the appropriate growth medium to form suspensions of $2-5 \times 10^4$ cells/ml. The suspensions were decanted into growth vessels and the cultures were equilibrated with a membrane filtered gaseous mixture of 5% CO_2 and 95% air, tightly sealed and

incubated at 37°C. When monolayers had formed, usually after 3-4 days, the growth medium was discarded and the cultures were rinsed with 20mls. of a solution of 0.025% trypsin in Earl's balanced salt solution, containing neither calcium nor magnesium ions. The cultures were incubated at 37°C in 5 mls. of the same trypsin solution for 20min. When the cell-cell and cell-substratum contacts had been disrupted the trypsin was neutralised by the addition of 45mls. of growth medium. The large clumps of cells which often remained after this treatment were dispersed by repeatedly passing the material through a wide bore (1.1mm internal diameter), sterile, stainless steel needle via a sterile plastic disposable syringe. The number of cells in suspension were estimated and portions were diluted to the appropriate concentration in growth medium, added to culture vessels, equilibrated with 5% CO₂ 95% O₂, sealed and stored as previously described.

Routinely, three types of culture vessels were used:

- 1) Roux cultures with a growing surface of 120cm² were seeded with 0.2-10x10⁶ cells in 100mls.;
- 2) Roller cultures in 'in vitro' bottles which provided a growing surface of 600cm² were seeded with 20x10⁶ cells in 150 mls. and rotated at 0.25 r.p.m. on a 'CEL-ROL' apparatus;
- 3) Petri dish cultures used for the measurement of ion fluxes, ouabain binding and Concanavalin A binding, provided a growing surface of 20cm² and were seeded with 0.05-1x10⁶ cells in 4 mls.

Measurement of cell number and volume. The routine estimation of the number and volume of cells in the suspensions obtained

from stock or experimental cultures was performed on the Coulter Counter (model ZF) linked to a Coulter Channalyser (model C1000). One ml. of cell suspension was diluted in 'Isoton' counting fluid and care was taken to obtain a concentration of cells unlikely to incur the errors of counting involved with coincidence, the passage of cells at a rate greater than the response time of the Coulter.

The Coulter counter withdraws a standard volume of 0.5 ml. through an orifice of internal diameter 100 μ m. Since the cell volume is small in relation to the orifice diameter, the alteration in resistance across the orifice, due to the volume of fluid displaced by the cell, is proportional to the cell volume. The counter emits voltage pulses, proportional in size to the resistance change, which are sorted by the Channalyser into 100 'Bins'. The system was routinely calibrated with latex spheres of known diameter and it was possible to obtain an absolute estimate of cell volume from the voltage distribution. The volume thus obtained was checked by comparison with the volumes calculated from measurements of diameter made using a calibrated micrometer eyepiece. Both of these methods assume that trypsinisation does not alter the cell volume but merely changes its shape (Lamb and Mackinnon 1971). For each sample the number of pulses in every tenth channel was recorded and the cell number and volume were computed from Simpson's rule of integration. In the case of cell suspensions which display a smooth log normal distribution, this method usually provides values to within 1% of those obtained when every

channel is considered (Aiton 1976). A typical volume distribution is shown in Fig. 1.1.

Chemical measurements. Protein was estimated by the method of Lowry (1951) with the modification that the material was first solubilised in 2.5% (w/v) sodium deoxycholate (Maddy and Spooner 1970). A standard curve of serum albumin against optical density at 700nm obtained by this method is displayed as Fig. 1.2. The relationship was reproducibly linear for quantities of protein up to 350ug/4mls.

Inorganic phosphate was determined by the method of Wahler and Wollenberger (1958) or by the method of Fiske and Subbarow (1925). Figures 1.3 and 1.4 respectively show the relationship between inorganic phosphate concentration and optical density obtained by these methods. The method of Wahler and Wollenberger proved the more sensitive and this method was employed where greater sensitivity was required.

Enzyme assays. 5'nucleotidase (E.C. 3.1.3.5.) was assayed as described by Lauter, Solyom and Trams (1972) and the inorganic phosphate released was determined by the method of Wahler and Wollenberger (1958).

Mg⁺⁺ATPase (E.C. 3.6.1.3.) and Na⁺+K⁺ stimulated ATPase (E.C. 3.6.1.4.) were assayed as follows: 0.2 mls. of sample were incubated with 1.4 mls. triethanolamine - HCl + 1mM MgCl₂ + 1mM ATP, pH 7.4 at 35°C for 30 min., with or without 60 mM NaCl + 10mM KCl. The activity was estimated by determination of the inorganic phosphate released. Those assays carried out in the absence of Na⁺ and K⁺ provided

Fig. 1.1 Volume distribution of Py 3T3 cells.

A cell plate containing 1.81×10^6 cells was trypsinised, neutralised and 'blasted' in the usual manner. 1.0mls. of the resulting suspension was diluted in 9.0mls. of 'Isoton' counting fluid. A 0.5ml. sample was drawn through the counting orifice of the Coulter counter(model ZF) and the resulting frequency distribution of pulse heights displayed on the Coulter Channalyser. Cell number and volume were calculated from Simpson's rule of integration, using every tenth channel. In this case the mean cell volume was $1.56 \times 10^{-3} \mu^3$.

FIG. 1.1

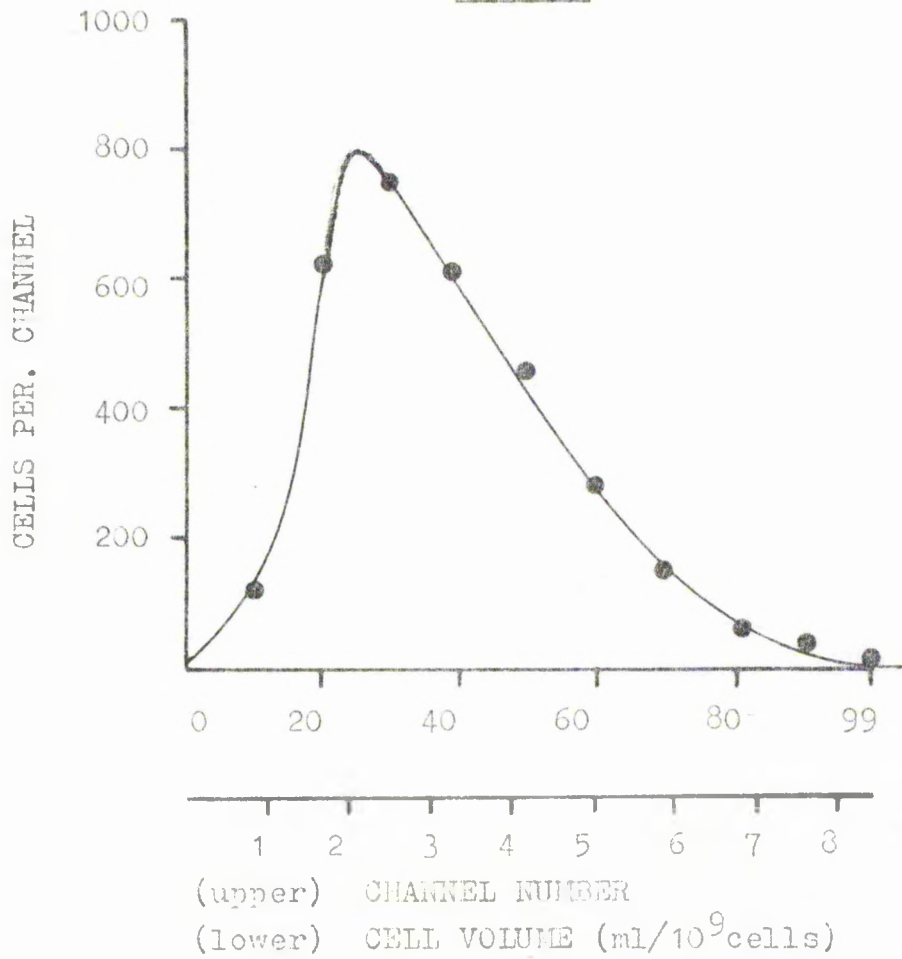


FIG. 1.2

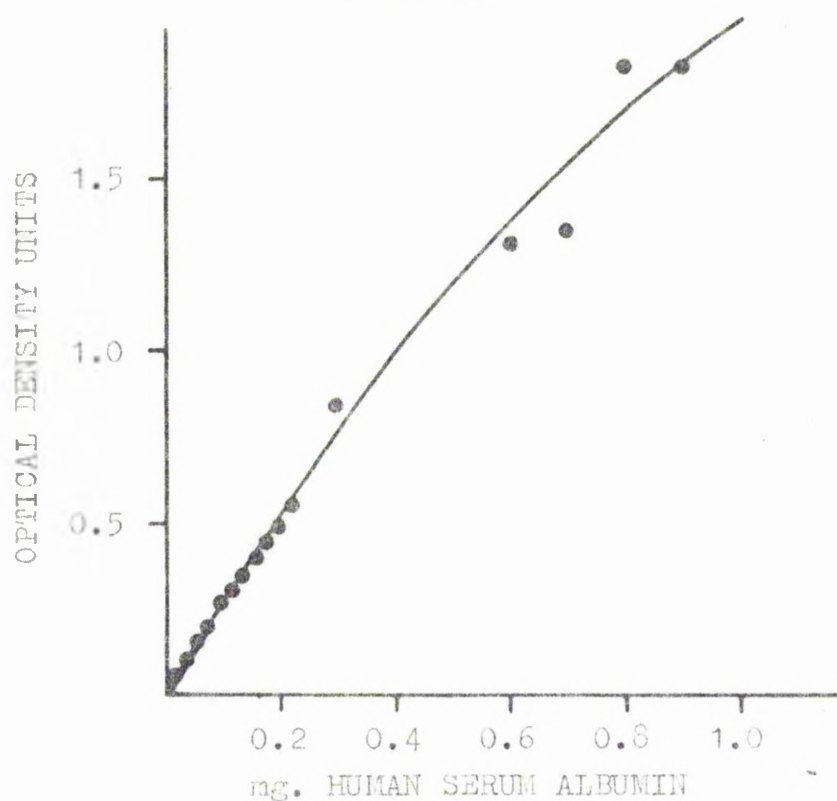


Fig. 1.2 Protein standard curve.

Protein was estimated by the method of Lowry et al with the modification that the material was first solubilised in 2.5(w/v) sodium deoxycholate. Human serum albumin was used as a standard.

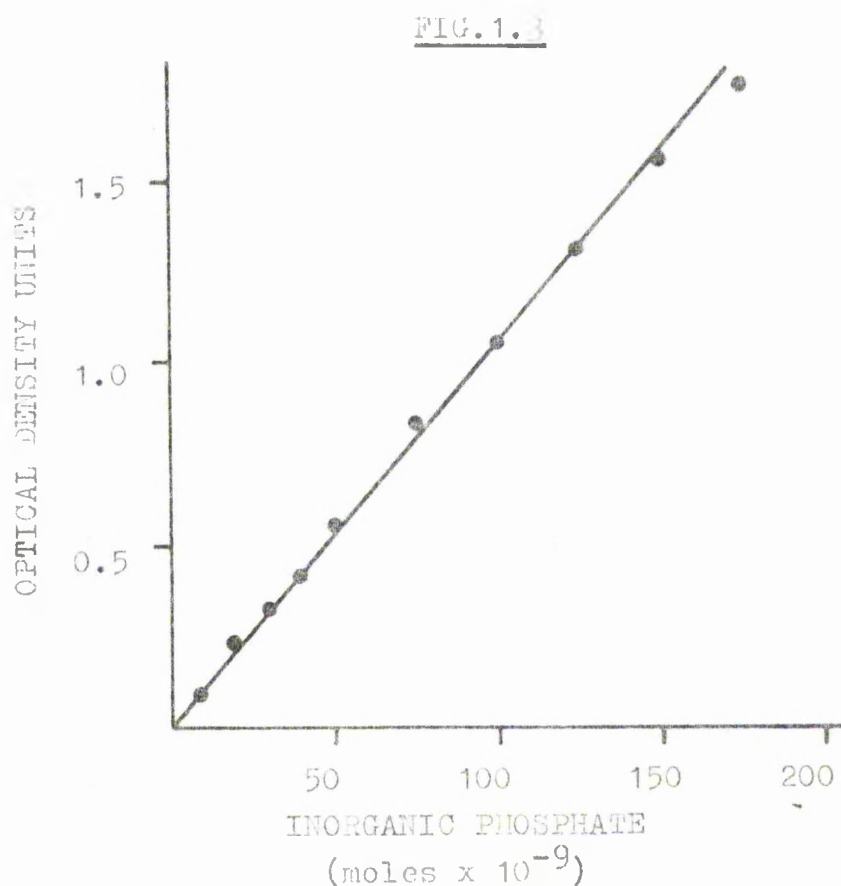


Fig. 1.3 The estimation of inorganic phosphate by the method of Wahler and Wollenberger.

Inorganic phosphate is expressed in terms of moles x 10^{-9} in a final volume of 1.6 ml. The relationship between optical density and phosphate concentration was reproducibly linear over the concentration range shown.

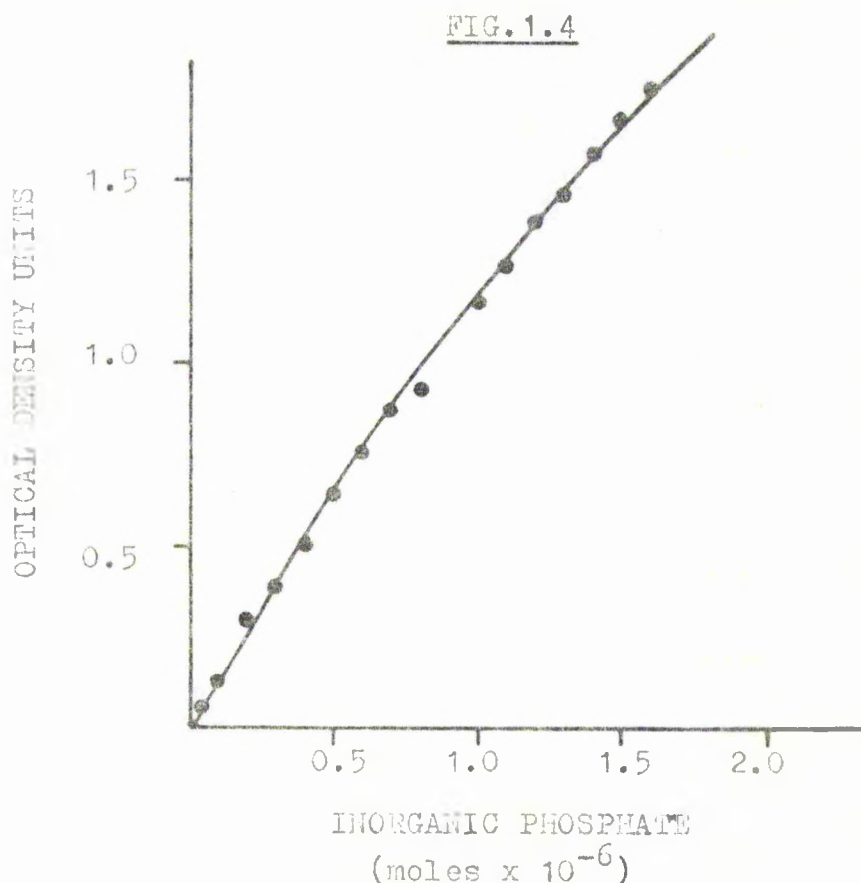


Fig. 1.4 The estimation of inorganic phosphate by the method of Fiske and Subarow.

Inorganic phosphate is expressed in terms of moles $\times 10^{-6}$ in a final volume of 1.6 ml. The relationship between optical density and phosphate concentration was reproducibly linear unto 0.7×10^{-6} moles per 1.6 ml. Care was taken to ensure that the optical density obtained from experimental readings was less than that obtained with 0.7×10^{-6} moles per 1.6 ml.

an estimation of the Mg^{++} ATPase activity which when subtracted from the values obtained in the presence of Na^+ and K^+ yielded an estimation of the activity of the $(Na + K)$ -ATPase. Blanks were run at the same time to measure the non enzymic hydrolysis of ATP and the sample phosphate content.

NADH and NADPH- ferricyanide reductase (E.C. 1.6.99.3. and 1.6.99.1.) were assayed according to the method of Zamudio et al (1969). Succinate dehydrogenase (E.C. 1.3.99.1.) was assayed by the method of King (1967).

Acid phosphatase (E.C. 3.1.3.2.) was determined by incubating the sample with 50 mM sodium succinate, pH 6.0, containing 5 mM p-nitrophenyl phosphate at $35^{\circ}C$ for 30 min. The samples were then cooled in ice and made alkaline by the addition of 1 ml. of 1M NaOH. The absorbance of the p-nitrophenol anion was measured at 410nm and corrected for non enzymic hydrolysis of the substrate and the absorbance of the sample material at 410nm.

Monoamine oxidase (E.C. 1.4.3.4.) was estimated by observing the decrease in optical density at 360nm on incubation of 0.2 mls. of the sample material in 3.0 mls. of 40 mM phosphate buffer, pH 7.4, containing 0.1 mM kynuramine.

Plasma membrane isolation. Since this chapter is concerned with the development of a gradient fractionation procedure much of the methodology must be discussed in the results section. For convenience, however, as much as possible will be outlined here.

SV40 3T3 cells were used to develop the isolation procedure which, it was hoped, could be successfully applied

to other cell types. These cells were chosen initially for their fragility hence ease of homogenisation and prolific growth; one roller culture provided sufficient material for a complete separation.

The cells to be used in plasma membrane isolation were cultured in roller bottles as previously described. When these cultures became confluent they were rinsed twice in homogenising buffer (10mM Tris-HCl, pH 7.4 or 8.0 at 4°C, containing Mg^{++} and/or Ca^{++} to a concentration of 0.5 to 2.5mM) and removed from the growing surface, with the aid of a rubber policeman, into 50 mls. of the same buffer. The suspension was centrifuged for 10 mins. at 3000xg at 4°C in a J20 rotor of a Beckman J21B centrifuge. The pellet of cells was resuspended in homogenising buffer to a final volume of 6 mls. and lysis was achieved by passing the material through a hypodermic syringe needle. Disruption was monitored by phase contrast microscopy and in the case of the 3T3 cell lines by the release of 5'-nucleotidase activity. A 19G Gillette needle, internal diameter 0.11mm length 4.0cms., was found to produce optimum disruption; use of a needle of internal diameter less than 0.08 mm resulted in the disruption of the nuclei while needles greater than 0.11mm resulted in inadequate disruption of the cells. Fig. 1.5 shows the effect of syringing SV40 3T3 cells through a 19G needle at the rate of 2-3 mls./sec on the activity of 5'-nucleotidase. 25 passages were required for the complete disruption of these cells, 35 passages for Py 3T3 cells, 45 for 3T3 cells and 60 for HeLa cells.

2 mls. of the homogenate were retained for the estimation

Fig. 1.5 The effect of homogenization on the specific activity of 5'-nucleotidase in SV40-3T3 cells.

As the cells are disrupted by repeated syringing the activity of the 5'-nucleotidase increases from 32 ± 3 to 109 ± 13 nM/mg/Hr. Prior to homogenization, the observed activity will be attributable to the exo-5'-nucleotidase activity and as the cells are disrupted, the intracellular activity will be observed. The activity reaches a maximum after 40 passages and at this point, 90% or more of the cells appear disrupted in phase contrast microscopy. ($n=2 \pm$ SEM).

Fig. 1.5

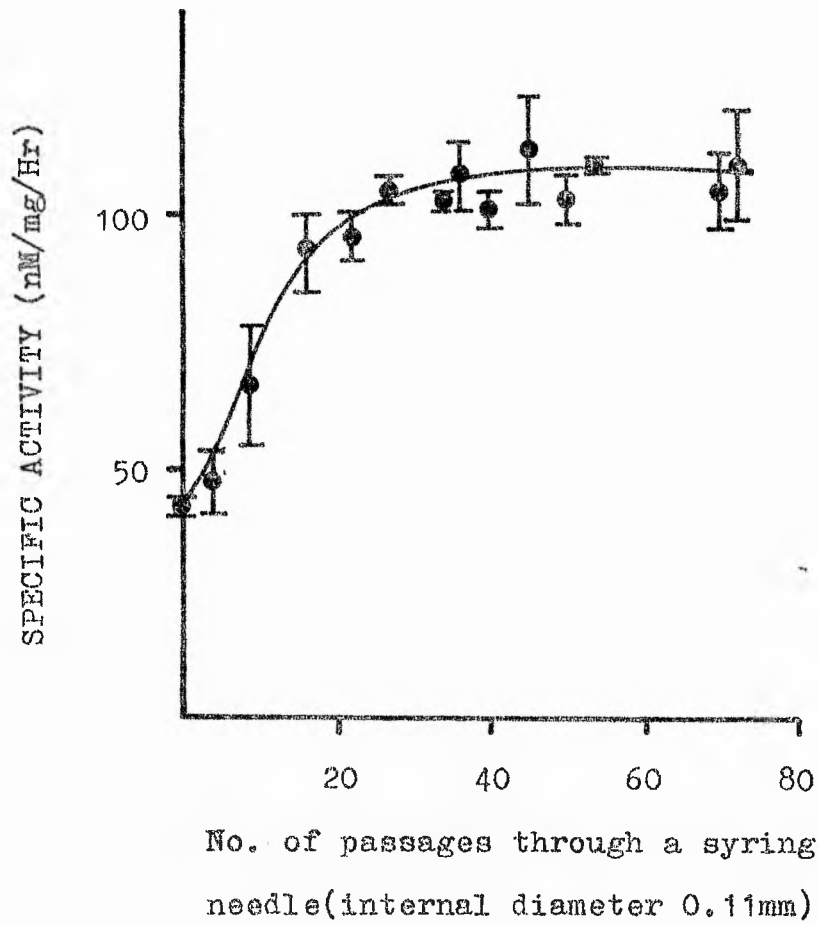


Fig. 1.5 The effect of homogenization on the specific activity of 5'-nucleotidase of SV40 3T3 cells.

of enzyme activity in the whole cell fraction and the remainder was adjusted to the required sucrose concentration (usually 40 or 55% w/w) by the gradual addition of a solution of sucrose 60% (w/w) in the homogenisation buffer. The resulting suspension was layered on a discontinuous sucrose gradient designed to separate the various cell organelles according to their equilibrium density (see Discussion).

Two gradients were employed; gradient I was of the following composition:-

Volume (mls.)	% sucrose (w/w in the homogenisation buffer)	density (g/cc at 4°C).
3	0	1.0
5	25	1.103
5	30	1.126
5	35	1.15
6	40	1.17
5	45	1.20
2	50	1.227
2	55	1.254
5	58	1.271

The above gradient was used when the sample was added as a 40% (w/w) sucrose solution. When the sample was adjusted to 55% (w/w) with respect to sucrose the gradient was altered; the sample was layered on the gradient between the 50 and 58% sucrose bands in a final volume of 7 mls. and the volumes of the 40 and 35% bands were reduced to 4 mls.

Gradient II was of the following composition:-

Volume (mls.)	% sucrose (w/w in the homogenisation buffer)	density (g/cc at 4°C)
6	0	1.0
7	35	1.15
7	37	1.165
7	45	1.20
3	50	1.227
3	55	1.254
5	58	1.271

The cellular material was layered on gradient II as a 7 ml. suspension 45% (w/w) with respect to sucrose.

The gradients were set up in 38 ml. cellulose nitrate tubes (of a Beckman SW-27 rotor) and centrifuged for 75 min. at 130 000xg and 4°C in a Beckman L265 B ultracentrifuge. The material which collected at each interface was labelled fraction I-V, according to its position on the gradient, and the material at the base of the tube was designated as the 'pellet'. A developed gradient together with the nomenclature used is shown in Plate 1.

The banded material was removed with a wide bore stainless steel needle; fraction I consisted of all the 0% sucrose band the first interface and half of the 25% sucrose band, fraction II consisted of the remainder of the 25% sucrose band the second interface and half of the 35% sucrose band and so on down the gradient. Each fraction was adjusted to 38 mls. by the addition of homogenisation buffer and centrifuged for 60 min. at 130 000xg and 4°C in the SW-27 rotor of a Beckman L2 65B centrifuge. This step was

PLATE I

The developed sucrose density gradient

Each interface was labeled as shown in the photograph.

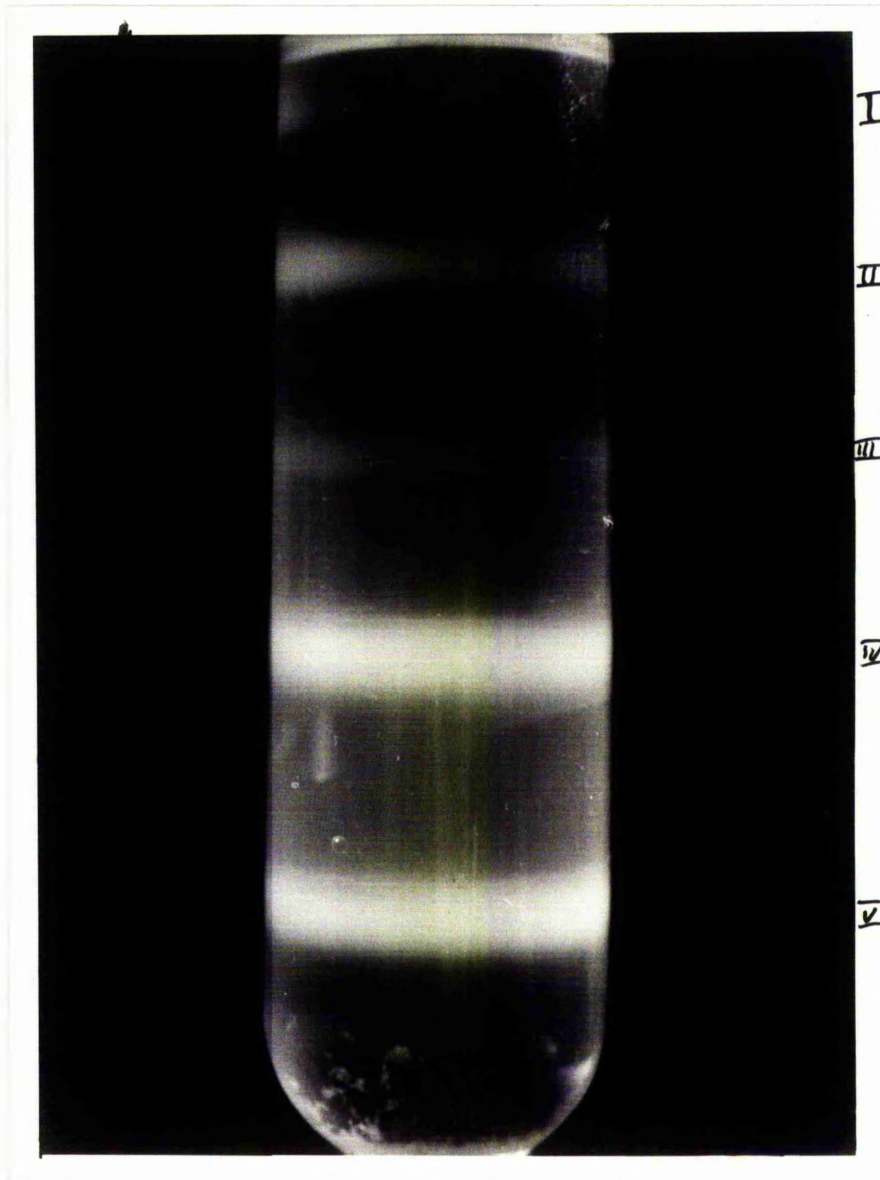


PLATE 1 The developed sucrose density
gradient.

included in order to remove the bulk of the sucrose which was found to interfere with the methods used for determination of protein, RNA and DNA. The pellet which formed during this time was adjusted to a known volume in homogenisation buffer and its enzymic and chemical properties were examined.

TABLE 1.A Dulbecco's Modification of Eagle's Medium (DMEM)

<u>Amino acids</u>	mg/l	<u>Vitamins</u>	mg/l
L-arginine. HCl	84.00	D-Ca pantothenate	4.00
L-cystine	48.00	Choline Chloride	4.00
L-glutamine	584.00	Folic acid	4.00
Glycine	30.00	i-inositol	7.00
L-histidine. HCl. H ₂ O	42.00	Nicotinamide	4.00
L-isoleucine	104.80	Pyridoxal. HCl	4.00
L-leucine	104.80	Riboflavin	0.40
L-lysine. HCl	146.20	Thiamine. HCl	4.00
L-methionine	30.00		
L-phenylalanine	66.00		
L-serine	42.00		
L-threonine	95.20		
L-tryptophan	16.00		
L-tyrosine	72.00		
L-valine	93.60		

<u>Inorganic salts and other components</u>	mg/l
NaCl	6400.00
KCl	400.00
MgSO ₄	98.00
CaCl ₂	200.00
NaH ₂ PO ₄ · H ₂ O	125.00
NaHCO ₃	3700.00
Fe(NO ₃) ₃ · 9H ₂ O	0.10
Na pyruvate	110.00
D-Glucose	4500.00
Phenol Red Na	15.90

Calf serum (10% v/v) and gentamicin (0.04 mg/ml) were added immediately prior to use.

TABLE 1.B Composition of Basal Medium Eagle with Earle's salts

<u>Amino acids (mg/L)</u>		<u>Vitamins (mg/L)</u>	
L-arginine	21.1	D-biotin	1.0
L-cystine	12.0	C-Capantothenate	1.0
L-glutamine	292.0	Choline chloride	1.0
L-histidine HCl	9.6	Folic acid	1.0
L-isolucine	26.2	I-inositol	2.0
L-leucine	26.2	Nicotinamide	1.0
L-lysine HCl	36.5	Pyridoxal HCl	1.0
L-methionine	7.5	Riboflavin	0.1
L-phenylalanine	16.5	Thiamine HCl	1.0
L-threonine	23.8		
L-tryptophan	4.0		
L-tyrosine	18.1		
L-valine	23.4		

Inorganic salts and other components (mg/L)

NaCl	6 800
KCl	400
CaCl ₂	200
MgSO ₄ · 7H ₂ O	98
NaH ₂ PO ₄	140
NaHCO ₃	1 680
D-glucose	1 000
Phenol red	10

Calf serum (10% v/v) and gentamicin (0.04 mg/ml) were added immediately prior to use.

RESULTS

Although there are problems with the use of 5'-nucleotidase as a plasma membrane marker (see Introduction) it was decided to use the enzyme in this role in order to develop the isolation procedure. This decision was made for several reasons: 1) the vesicles produced by syringing were morphologically indistinguishable from those of the E.R.; 2) during initial experiments with SV 3T3 cells no (Na+K)-ATPase activity could be detected in any of the fractions, and 3) at least 30 \pm 3% of the 5' nucleotidase activity could be detected in intact cells and must therefore be associated with the plasma membrane, the cell surface being impermeable to substrate. Homogenisation increases the measurable activity of SV 3T3 cells from 32 \pm 3 to 109 \pm 13 nm/mg/Hr (Fig. 1.5).

DIVALENT CATIONS

There is evidence to suggest that the divalent cation regime may effect the recovery of 5'-nucleotidase (Neville 1976) especially in the lower density fractions (Bingham and Burke 1972). Experiments were therefore conducted to investigate the effect of divalent cation concentration on the distribution of 5'-nucleotidase in the gradients. The concentration of divalent cations was limited to the range 2.5 to 0.5mM since beyond these extremes the nuclei either clump, trapping cellular components, or lyse, causing nonspecific membrane aggregation. NADH-ferricyanide reductase and succinate

dehydrogenase activities were also measured to monitor the distribution of the E.R. and Mitochondria.

The distribution of the relative specific activity of these enzymes is shown in Figs. 1.6, 1.7 and 1.8 respectively.

The effects of 5 divalent cation concentrations were examined. In each experiment the same divalent cation concentration was used throughout the isolation procedure. These concentrations are referred to in the illustrations in the following manner:

<u>Mg⁺⁺ (mM)</u>	<u>Ca⁺⁺ (mM)</u>	<u>Label</u>
2.0	0.5	(i) a
0.5	0.5	(i) b
--	0.5	(i) c
2.0	--	(ii) a
1.0	--	(ii) b

The observations directly related to the plasma membrane isolation are as follows:-

Fractions II, III and IV in most of the gradients contained the highest relative specific activities of 5'-nucleotidase (Fig. 1.6(i) and (ii)). The greatest relative specific activity of this enzyme occurred in fraction II from gradients containing 1mM MgCl₂ (Fig. 1.6(ii)b). This fraction also contained low levels of rotenone insensitive NADH-ferricyanide reductase (Fig. 1.7(ii)b) and succinate dehydrogenase (Fig. 1.8(ii)b). Gradient (ii)b therefore afforded the best plasma membrane isolation, based on these criteria.

Some secondary observations, to be discussed below, may prove useful in the isolation of other cell organelles, but are not of immediate relevance to the isolation of a plasma membrane fraction. The results directly concerning the isolation of plasma membrane material are continued on p.49 with the effect of Potter homogenization on the distribution of marker enzymes.

5'-Nucleotidase. Many authors consider this enzyme to be an adequate marker for the plasma membrane. Its recovery was affected by alterations in the divalent cation concentration. The general effect of a reduction in the divalent cation concentration was an increase in relative specific activity. Fig. 1.6(i) shows the effect of a reduction at a constant (0.5mM) calcium level. A decrease from 2.0mM MgCl_2 to 0.5mM MgCl_2 (Fig. 1.6(i) a and b) resulted in an increase in all of the fractions except II and pellet. The activity of fraction II was increased in the absence of MgCl_2 (Fig. 1.6 (i) c). The distributions in gradients containing 2.0mM MgCl_2 with and without CaCl_2 (Fig. 1.6(i) a and (ii) a) were not significantly different except for a slight increase in fraction II in the latter. A further reduction in MgCl_2 to 1.0mM (Fig. 1.6(ii) b) resulted in an increase in the relative specific activity of all the fractions except V which was unaltered.

NADH-Ferricyanide reductase. The highest relative specific activity for this enzyme (considered to be a marker for smooth E.R.) was found in fraction IV in each gradient

Fig. 1.6(i) The distribution of 5'-nucleotidase activity in gradients containing (a) 2.0mM MgCl_2 + 0.5mM CaCl_2 , (b) 0.5mM MgCl_2 +0.5mM CaCl_2 , and (c) 0.5mM CaCl_2 .

When the divalent cation concentration was altered from 2.5mM to 1.0mM, the relative specific activities of fractions III, IV and V were significantly increased and the relative specific activity (R.S.A.) of fraction II was significantly reduced. A further reduction to 0.5mM CaCl_2 resulted in a significant increase in fraction II and decreases in fractions V and pellet. The activities of all of the fractions, except the pellet, from gradient (c) were significantly greater than the corresponding fractions from gradient(a).

The highest R.S.A. of these experiments was found in fractions II and III of gradient(c). However, in this gradient the nuclei were unstable and largely fragmented.

The columns are the means of 5-10 duplicate observations and the vertical bars represent the standard error of the mean, n.d.= not done.($p < 0.05$ by students T test for all the alterations quoted above.)

Fig. 1.6(i)

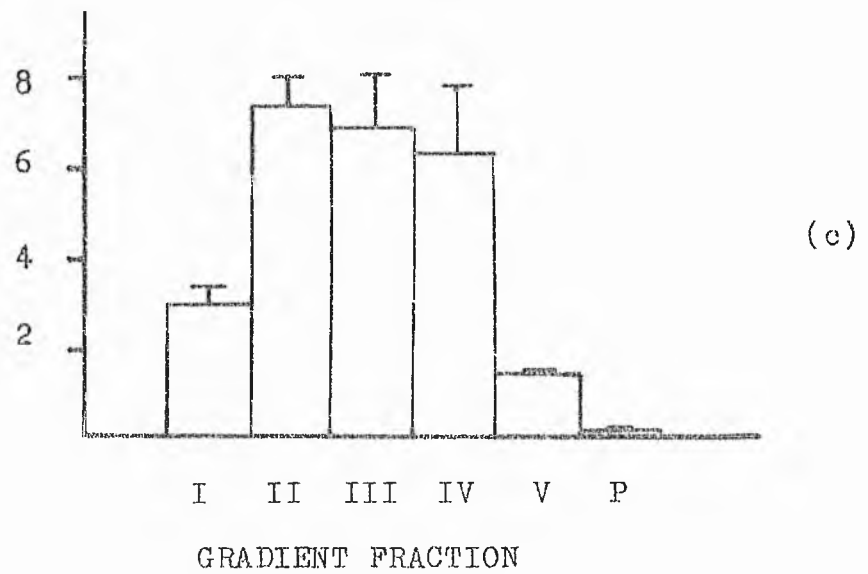
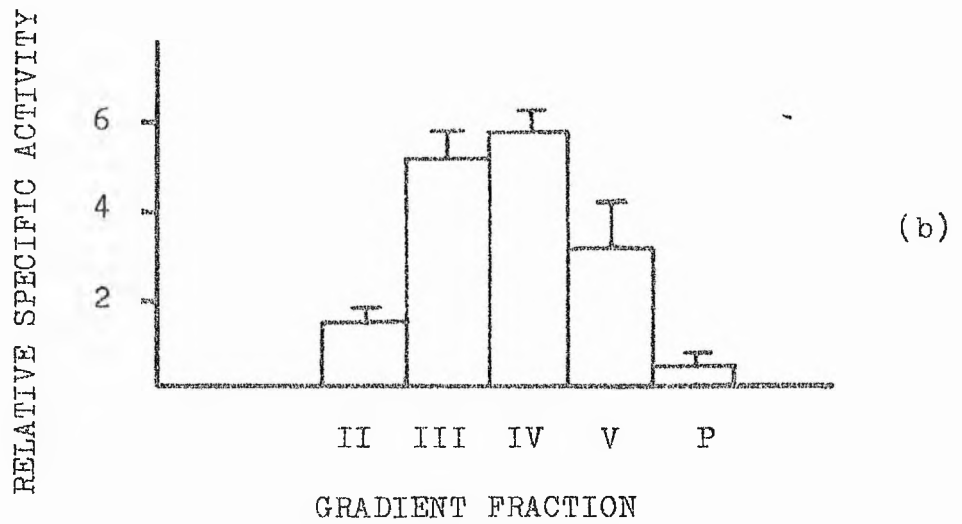
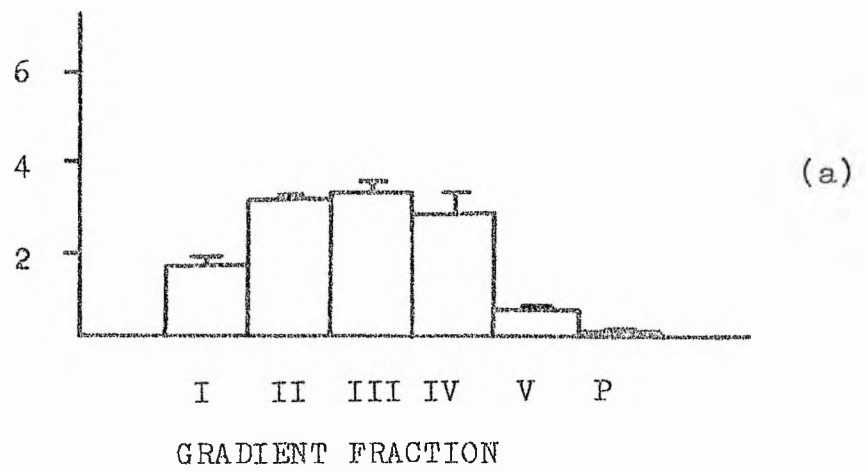


Fig. 1.6(ii) The distribution of 5'-nucleotidase activity in gradients containing (a) 2.0mM MgCl_2 and (b) 1.0mM MgCl_2 .

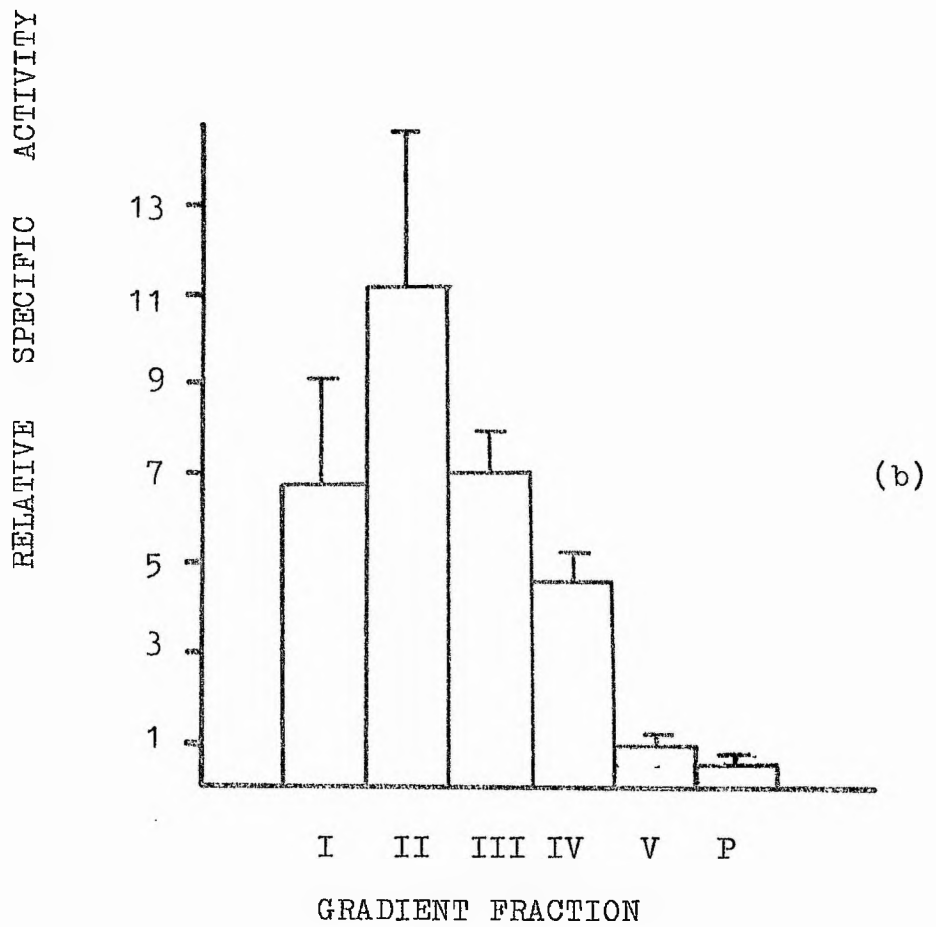
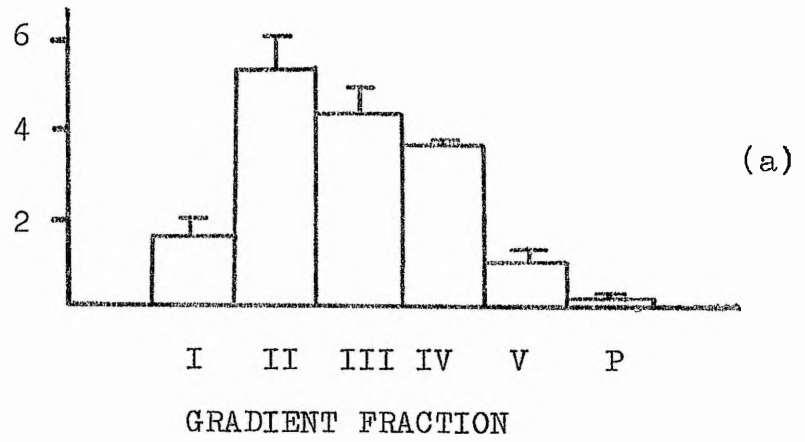
When Ca^{++} was omitted from gradient 1.6(i)a there was a significant increase in the 5'-nucleotidase activity of fractions II (from 3.1 ± 0.1 to 5.3 ± 0.8) and III (from 3.2 ± 0.2 to 4.3 ± 0.5 ; $p=0.05$ in both cases).

When the divalent cation concentration was further reduced to 1.0mM Mg^{++} the R.S.A of fraction II increased significantly (from 5.3 ± 0.8 to 11.2 ± 3.5 ; $p < 0.05$). The activities of the other gradient fractions, with the exception of V, also increased significantly.

The highest R.S.A of all fractions was found in fraction II of gradients containing 1.0mM MgCl_2 .

The columns represent the mean of 4-7 duplicate observations and the vertical bars represent the standard error of the mean.

Fig. 1.6(ii)



(Fig. 1.7(i) and (ii)). When the Mg^{++} concentration was reduced in the presence of a constant (0.5mM) Ca^{++} concentration (Fig. 1.7(i) a and b) the relative specific activity of fractions III and IV increased. The omission of $MgCl_2$ from the latter gradient resulted in a decrease in the relative specific activity of fractions III, IV, V and pellet. As was the case with 5'-nucleotidase, there was no significant difference between fractions isolated in the presence of 2.0mM $MgCl_2$ with and without 0.5mM $CaCl_2$ (Fig. 1.7(i) a and (ii) a). A reduction of $MgCl_2$ to 1.0mM (Fig. 1.7(ii) b) brought about an increase in the relative specific activity of the enzyme in fractions III, IV, V and pellet.

Succinate Dehydrogenase. This enzyme is considered to be a marker for the inner mitochondrial membrane. In the presence of both $MgCl_2$ and $CaCl_2$ (Fig. 1.8(i) a and b) there were two peaks of activity; a low density peak, fractions I and II, and a high density peak, fractions V and pellet. With 0.5mM $CaCl_2$ alone (Fig. 1.8(i) c) the distribution was quite different. There was either a redistribution of activity or an inhibition of the enzyme in the lower density fractions. Fraction V in the latter gradient had a significantly greater relative specific activity of succinate dehydrogenase than gradient 1.6(i) a and b. With the exception of fraction I, the profile of the relative specific activity of succinate dehydrogenase was the same with 2.0mM $MgCl_2$ in the presence and absence of 0.5mM $CaCl_2$ (Fig. 1.8(i) a and (ii) a). A reduction of $MgCl_2$ concentration from 2.0mM to 1mM resulted in a loss of activity of

Fig. 1.7(i) The distribution of rotenone insensitive NADH-ferricyanide reductase activity in gradients containing (a) 2.0mM MgCl_2 + 0.5mM CaCl_2 , (b) 0.5mM MgCl_2 + 0.5mM CaCl_2 and (c) 0.5mM CaCl_2 .

The R.S.A of none of the fractions from gradient (a) was significantly greater than 1.

A reduction in divalent cation concentration to 0.5mM MgCl_2 + 0.5mM CaCl_2 resulted in significant increases in the activities of fractions III and IV (from 1.2 ± 0.3 to 2.7 ± 0.7 and from 1.5 ± 0.3 to 3.0 ± 0.4 respectively; $p < 0.05$ in both cases). The remainder of the gradient was unaltered.

In gradients containing 0.5mM CaCl_2 alone, all of the fractions had relative specific activities of less than 1 and significantly less than the corresponding fractions from gradient i(b).

The columns are the mean of 5-8 experiments performed in duplicate and the vertical bars represent the standard error of the mean.

Fig. 1.7(i)

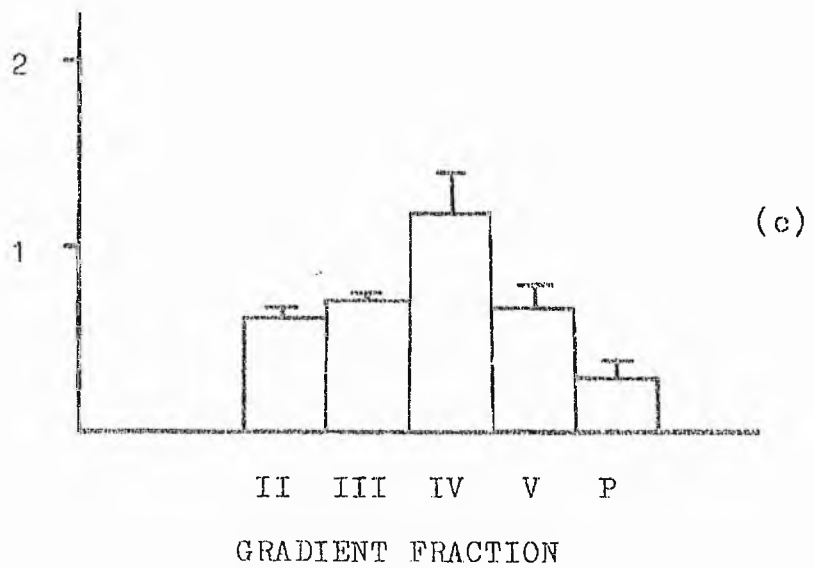
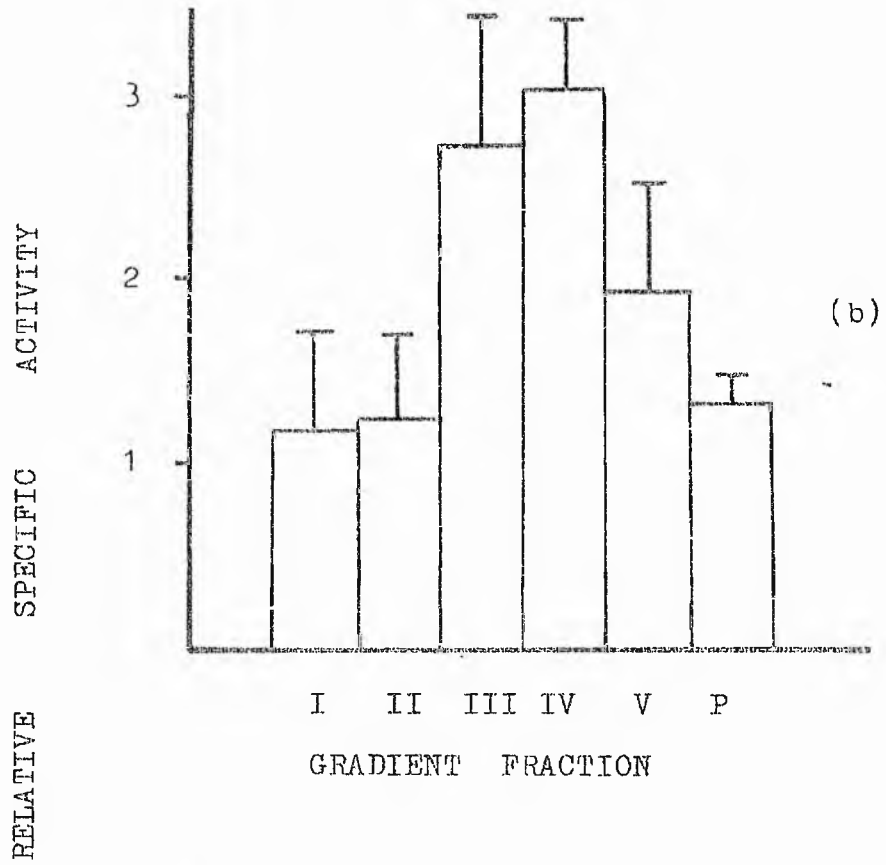
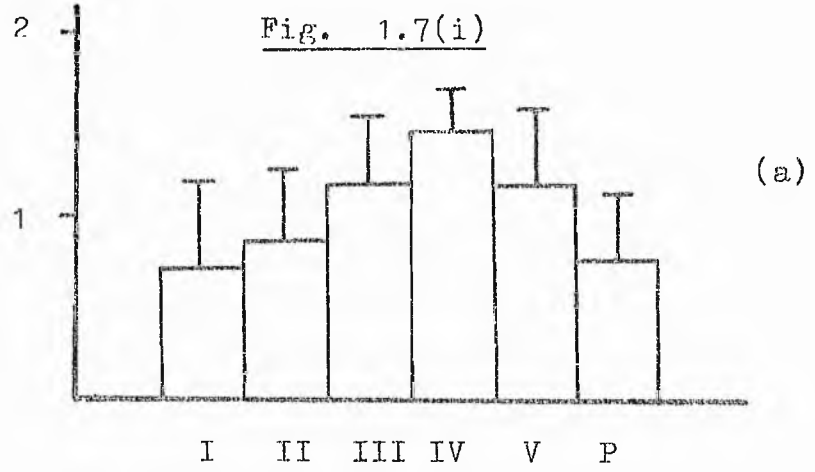


Fig. 1.7(ii) The distribution of rotenone insensitive NADH-ferricyanide reductase in gradients containing (a) 2.0mM MgCl_2 and (b) 1.0mM MgCl_2 .

In the presence of 2.0mM MgCl_2 each of the fractions, except IV, had an R.S.A of less than 1 and the R.S.A. of fractions I, IV, V and pellet were less than in gradient (i)a.

In the presence of 1.0mM MgCl_2 the R.S.A of each of the fractions was significantly greater than the corresponding fractions from gradients containing 2.0mM MgCl_2 .

The columns represent the mean of 5-8 experiments performed in duplicate and the vertical bars represent the standard error of the mean.

Fig. 1.7(ii)

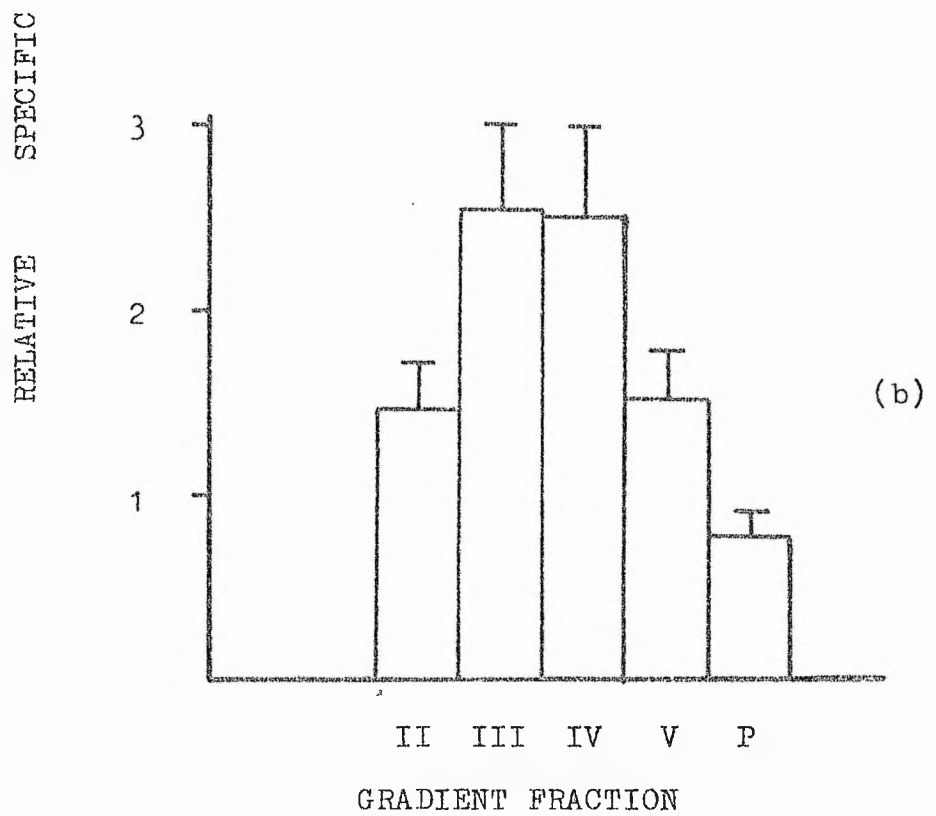
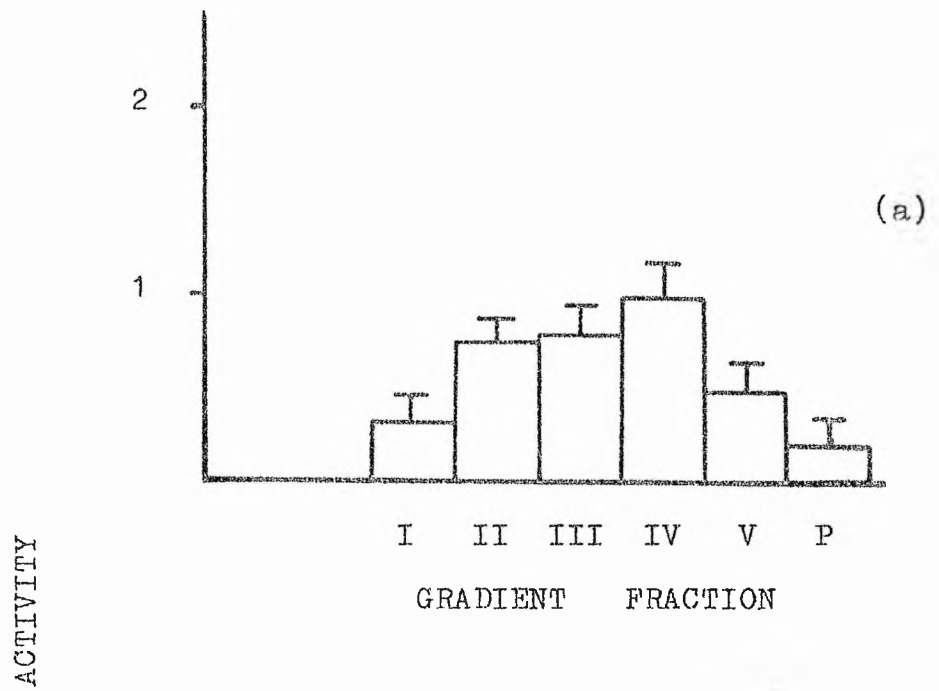


Fig. 1.8(i) The distribution of succinate dehydrogenase activity in gradients containing (a) 2.0mM MgCl_2 + 0.5mM CaCl_2 , (b) 0.5mM MgCl_2 + 0.5mM CaCl_2 and (c) 0.5mM CaCl_2 .

In gradient (i)a only fractions I and II possessed R.S.A.s greater than 1. When the divalent cation concentration was reduced to 1.0mM the R.S.A of the nuclear fraction was significantly increased (from 1.3 ± 0.3 to 2.4 ± 0.6 ; $p=0.05$).

In the presence of 0.5mM CaCl_2 alone, no activity could be detected in fractions I or II. Under these conditions the R.S.A.s of fractions III and IV were significantly greater than the corresponding fractions of gradient (i)b (0.9 ± 0.2 c.f. 1.6 ± 0.1 and 1.2 ± 0.4 c.f. 2.2 ± 0.4 respectively; $p < 0.05$).

The columns represent the mean of 5-7 experiments performed in duplicate and the vertical bars represent the standard error of the mean.

Fig. 1.8(i)

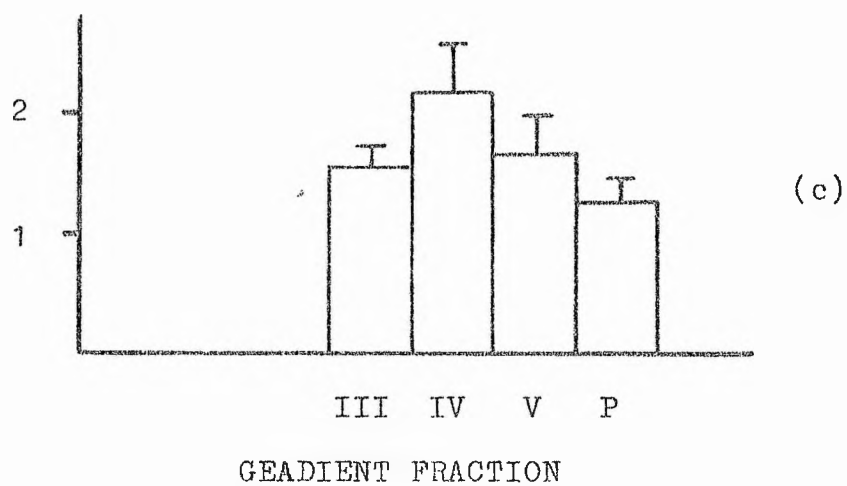
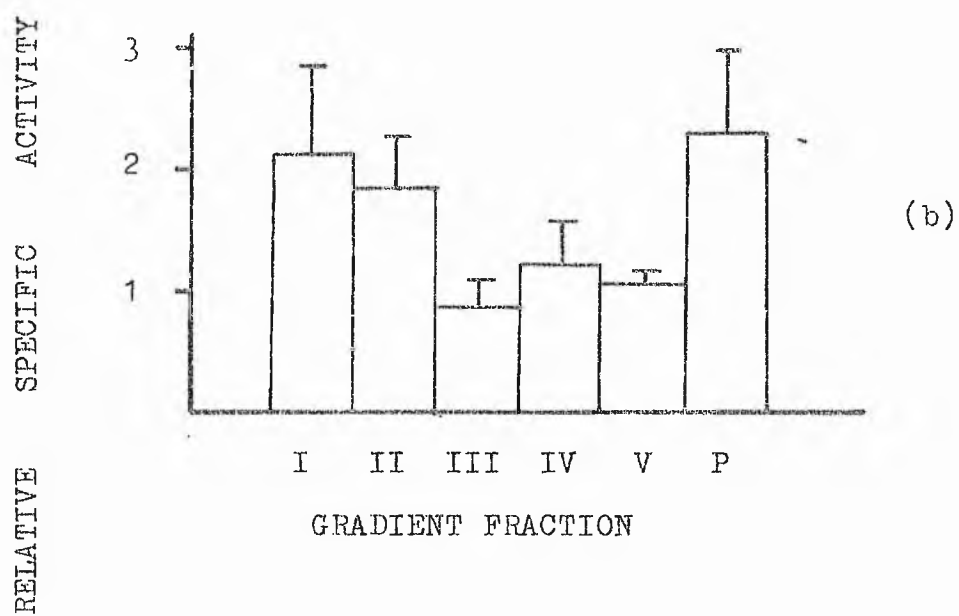
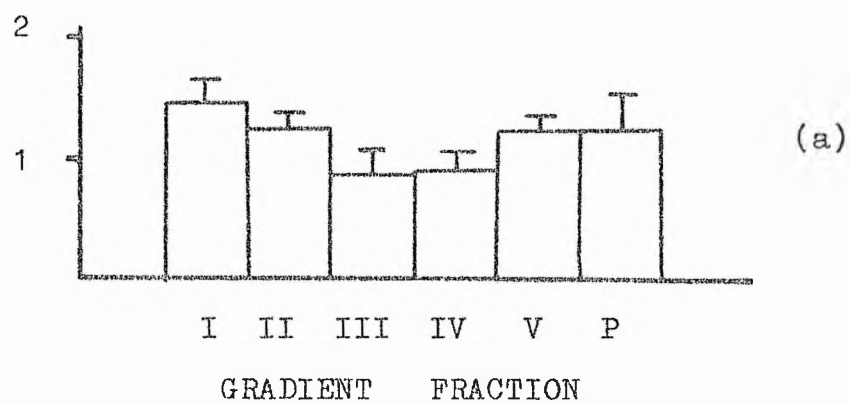


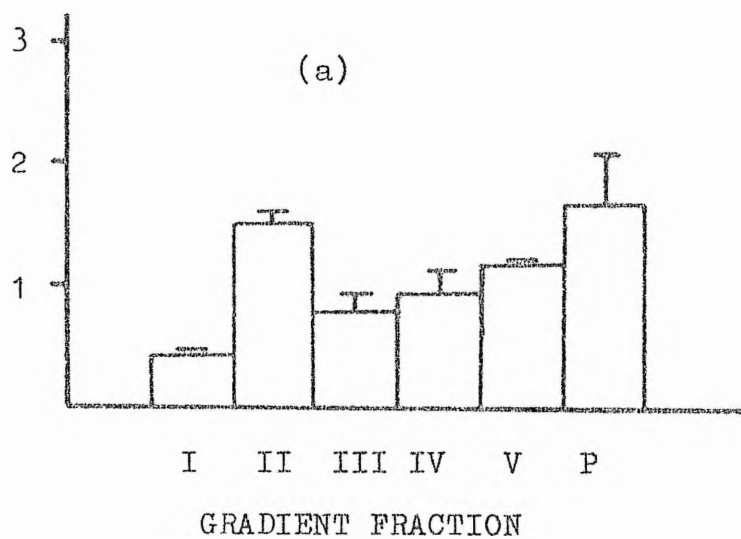
Fig. 1.8(ii) The distribution of succinate dehydrogenase activity in gradients containing (a) 2.0mM MgCl_2 and (b) 1.0mM MgCl_2 .

Except for fraction I, the R.S.A.s of the corresponding fractions of gradients (i)a and (ii)a were not significantly different. Fraction I decreased significantly as the divalent cation concentration was reduced.

When the divalent cation concentration was further reduced to 1.0mM MgCl_2 , succinate dehydrogenase activity could not be observed in fraction I or II and the activities of fractions III and IV were significantly increased (from 0.8 ± 0.1 to 1.6 ± 0.2 and from 0.95 ± 0.2 to 3.8 ± 0.7 respectively; $p < 0.05$). Fraction IV from gradient (ii)b possessed the highest relative specific activity obtained in these experiments.

The columns represent the mean of 6-9 observations performed in duplicate and the vertical bars represent the standard error of the mean.

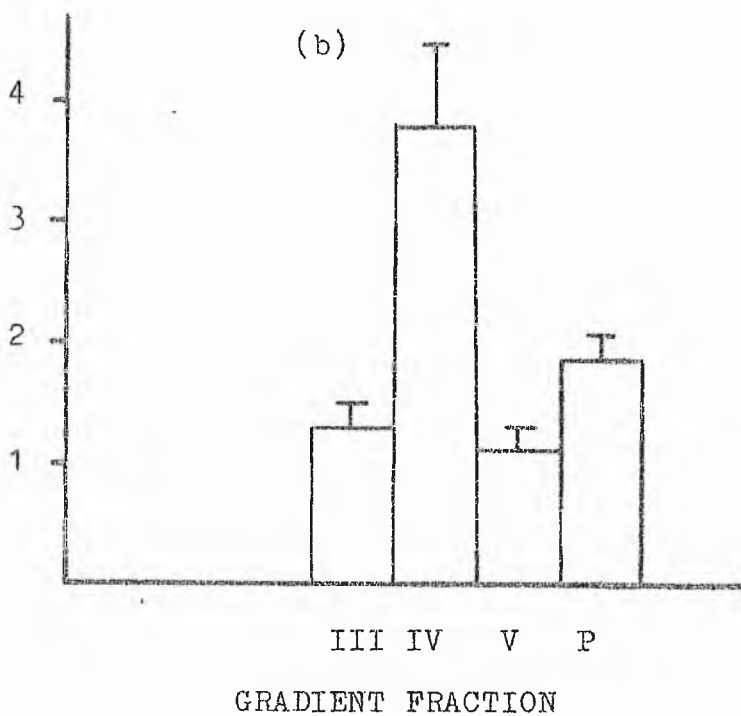
Fig. 1.8(ii)



ACTIVITY

SPECIFIC

RELATIVE



GRADIENT FRACTION

fractions I and II and an increase in the activity of fractions III and IV.

Percentage protein recovery. The relative specific activity of an enzyme is a composite of the percentage recovery of protein and enzyme in a given fraction. Table 1.1 illustrates the effect of divalent cations on the distribution of protein in the gradients. Columns (i)a, (i)b, (i)c, (ii)a and (ii)b represent the distribution of protein in gradients of 2.0mM MgCl_2 + 0.5mM CaCl_2 , 0.5mM MgCl_2 + 0.5mM CaCl_2 , 0.5mM CaCl_2 , 2.0mM MgCl_2 and 1mM MgCl_2 respectively. The total protein recovered was reduced from around 100% at concentration greater than 2.0mM to around 30% at concentrations of less than 1.0mM. It is not possible to state whether the reduction is due to a solubilisation of loosely bound protein or to a decrease in the binding of cytoplasmic constituents. Both of these processes might be expected with a reduction in divalent cation concentration. The reduction in protein with divalent cation is associated with a reduced recovery from fraction V.

Percentage 5' nucleotidase recovery. The percentage distribution of 5'-nucleotidase also varies depending on the divalent cation concentration (Table 1.2). When the divalent cation concentration is reduced below 1.5mM the total recovery of this enzyme is decreased from a value not significantly less than 100%, of the total activity added to the gradient, to 85% in the case of 1.0mM MgCl_2 and 75% in the case of 0.5mM CaCl_2 . In the presence of both Ca^{++} and Mg^{++} there were no significant trends in any of the

TABLE 1.1 The recovery of protein from gradients containing (i)a 2.0mM $MgCl_2$ + 0.5mM $CaCl_2$, (i)b 0.5mM $MgCl_2$ + 0.5mM $CaCl_2$, (i)c 0.5mM $CaCl_2$, (ii)a 2.0mM $MgCl_2$, and (ii)b 1.0mM $MgCl_2$.

As the divalent cation concentration is reduced below 2.0mM the total recovery of protein is greatly reduced. Most of the reduction in protein recovered, appears to be associated with decreases in fraction V. The protein recovered in fractions IV, V and pellet of gradient (i)b is less than that recovered in the same fractions of gradient (i)a. The only significant difference between gradients (i)b and (i)c involves fractions V and pellet, the protein contents of which increase and decrease respectively, as the divalent cation concentration is reduced.

Fractions I and II from gradient (ii)a had higher protein contents than the corresponding fractions from gradient (i)a and this may explain some of the slight increase in protein recovered from the former gradient. When the divalent cation concentration is further reduced to 1.0mM the protein content of all of the gradient fractions is reduced, so that the total protein recovered from the gradient is only some 40% of that applied.

The values are the mean of 5-10 experiments performed in duplicate \pm the standard error of the mean.

<u>Gradient fraction</u>	<u>(i)a</u>	<u>(i)b</u>
I	2.3 ± 0.5	1.6 ± 0.7
II	3.4 ± 1.4	2.4 ± 0.6
III	6.0 ± 2.9	4.3 ± 0.8
IV	11.0 ± 2.1	3.8 ± 1.1
V	43.0 ± 4.3	11.5 ± 2.7
Pellet	22.2 ± 5.2	10.0 ± 3.0
Total	83.0 ± 1.0	34.0 ± 3.0

TABLE 1.1

<u>(i)c</u>	<u>(ii)a</u>	<u>(ii)b</u>
1.1 ± 0.4	7.2 ± 0.3	1.4 ± 0.2
2.2 ± 1.2	6.1 ± 0.2	1.4 ± 0.5
3.1 ± 1.2	9.1 ± 0.2	2.6 ± 0.7
3.5 ± 0.9	9.5 ± 1.4	3.6 ± 0.8
6.8 ± 0.3	39.4 ± 12.0	19.2 ± 3.9
24.6 ± 10.0	26.2 ± 16.0	8.9 ± 3.6
42.0 ± 8.0	97.0 ± 3.0	37.0 ± 8.0

Table 1.2 The effect of divalent cation concentration on the percentage recovery of 5'-nucleotidase in the various gradient fractions.

In all of the gradients except (i)c the recovery of the enzyme is not significantly less than 100%. In gradient (i)c the recovery is only $80 \pm 3\%$ and the loss of activity is associated with a reduction in the recovery in fraction V.

A decrease in divalent cation concentration from (i)a to (i)b brings about a decrease in the percentage recovered in fraction II and an increase in fraction V. A further decrease in divalent cation concentration to 0.5mM CaCl_2 brings about an increase in fraction II and a decrease in fraction V. In gradient (ii)a the percentage of the enzyme recovered in fractions I and II is greater than in the same fractions from gradient (i)a. When the divalent cation concentration is further reduced to 1.0mM MgCl_2 , the percentage of the enzyme recovered in fraction V is significantly reduced.

The gradients are labeled as Table 1.1. The values are the means of 5-10 experiments \pm the standard error of the mean, n.d.= not done. $p < 0.05$ by students T test for all of the alterations quoted above.

<u>Gradient fraction</u>	<u>(i)a</u>	<u>(i)b</u>
I	3.8 ± 0.5	n.d.
II	10.5 ± 2.5	3.6 ± 0.7
III	19.5 ± 5.5	22.0 ± 3.0
IV	30.6 ± 4.4	25.4 ± 4.0
V	28.9 ± 3.0	44.4 ± 6.7
Pellet	4.0 ± 0.7	4.9 ± 1.9

TABLE 1.2

<u>(i)c</u>	<u>(ii)a</u>	<u>(ii)b</u>
3.8 ± 1.0	8.1 ± 1.1	9.3 ± 2.0
15.4 ± 4.8	22.1 ± 1.9	15.8 ± 4.4
21.8 ± 5.3	27.0 ± 1.9	18.5 ± 3.2
22.6 ± 4.5	23.6 ± 2.0	16.8 ± 2.5
10.5 ± 0.5	28.4 ± 6.3	20.9 ± 3.2
2.5 ± 0.7	3.9 ± 1.6	4.9 ± 1.9

fractions as the divalent cation concentration was reduced. While the total recoveries in gradients (i)a and (i)b are not significantly less than 100% fraction V of the former was greater than the latter and fraction II of the former was less than that of the latter. The 25% reduction in total recovery in gradient (i)c is associated with a large decrease in the percentage located in fraction V; in all other respects there is no significant difference between gradients (i)a and (i)c. In gradient (ii)a the percentage recovery of the enzyme in fractions I and II is significantly greater than in the same fractions from gradient (i)a while none of the other fractions alter significantly. The 15% decrease in total activity recovered from gradient (ii)b is associated with a reduction in the enzymic activity in fractions III and IV; in all other respects gradients (ii)a and (ii)b are not significantly different.

Percentage rotenone insensitive NADH-ferricyanide reductase activity

The total recovery of this enzyme decreased with the reduction in divalent cation concentration (Table 1.3). The recovery in gradient (i)a (2.0mM $MgCl_2$ + 0.5mM $CaCl_2$) was not significantly different from 100% and was reduced to around 54% in gradients (i)b, (i)c and (ii)a. Gradient (ii)b had the lowest recovery of this enzyme. The highest percentage activity of this enzyme was found in fraction V in all gradients except (ii)b. Since fraction V of this gradient contained 18% less NADH-ferricyanide reductase than fraction V of gradients (i)b, (i)c and (ii)a and fraction V of (i)a contained 30% more activity than gradients b, c and d, it would be convenient if the differences in fraction V could account for the

TABLE 1.3 The effect of divalent cation concentration on the percentage recovery of rotenone insensitive NADH-ferricyanide reductase activity.

The total recovery from the gradient decreases with decreasing divalent cation concentration, from $97 \pm 10\%$ of that applied to gradient (i)a to $53 \pm 4\%$ in gradients (i)b, (ii)a and (ii)b (the individual values were not significantly different) to $23 \pm 5\%$ in gradient (i)c. The reduction in recovery is associated with significant decreases in the activity recovered in fraction V.

The only significant difference between gradients (i)a and (i)b is the reduced recovery of activity in fraction V of the latter. As the divalent cation concentration is further reduced, to 0.5mM Ca^{++} ((i)c), the activity of all the gradient fractions is reduced. In gradient (ii)a the activities in fractions IV, V and pellet are significantly less than in the same fractions of (i)a. When the divalent cation concentration is further reduced to 1.0mM MgCl_2 , the enzyme activity in fractions I and II is reduced while none of the other gradient fractions is altered significantly.

TABLE 1.3

<u>Gradient fraction</u>	(i)a	(i)b	(i)c	(ii)a	(ii)b
I	1.4 \pm 0.6	1.9 \pm 0.7	n.d.	2.4 \pm 0.6	n.d.
II	2.9 \pm 0.9	3.0 \pm 0.8	1.4 \pm 0.4	4.7 \pm 0.5	2.0 \pm 0.4
III	7.0 \pm 2.3	11.7 \pm 2.2	2.2 \pm 0.5	7.4 \pm 0.8	6.6 \pm 1.2
IV	16.2 \pm 2.3	12.2 \pm 1.7	4.1 \pm 0.8	9.5 \pm 1.3	8.8 \pm 1.5
V	50.3 \pm 10.6	22.5 \pm 4.9	4.7 \pm 0.5	20.1 \pm 5.0	29.2 \pm 4.5
Pellet	17.2 \pm 5.1	13.7 \pm 2.8	7.9 \pm 2.3	5.8 \pm 3.2	7.0 \pm 1.8

altered total recovery. In gradients (i)a, (i)b and (i)c the percentage of the enzyme in the nuclear fraction was reduced as the divalent cation concentration was reduced, while in gradients (i)a and (i)b there was no difference in these fractions.

Percentage Succinate dehydrogenase recovery. The distribution and recovery of succinate dehydrogenase in gradients of different divalent cation concentration is shown in Table 1.4. The nomenclature in this case was the same as the previous three tables. In fractions I and II of gradients (i)c (0.5mM CaCl_2) and (ii)b (1mM MgCl_2) the levels of succinate dehydrogenase were below the limits of detection. There was an apparent decrease in total activity recovered as the divalent cation concentration was reduced. At concentrations greater than 2.0mM the recovery was not significantly less than 100% while at concentrations below 2.0mM the recovery was reduced to around 50%. The greatest percentage recovery in each gradient was found in fraction V. Most of the loss of activity appears to be due to a reduction in the recovery in this fraction.

It would appear from the data presented above that the optimum plasma membrane isolation was achieved in gradients containing 1mM MgCl_2 . Fraction II of this gradient contains 5'-nucleotidase at a specific activity 11 fold greater than that of the whole cell homogenate. In this fraction there was no detectable succinate dehydrogenase activity and a two fold increase in the activity of rotenone insensitive NADH-ferricyanide reductase.

TABLE 1.4 The effect of divalent cation concentration on the recovery of succinate-ferricyanide reductase activity from the various gradient fractions.

The total recovery from gradients (i)a and (ii)a was not significantly less than that applied and the overall distribution in the two gradients was similar. The total recoveries from the other gradients were approximately 50% of the activities applied. These reductions were associated with decreases in the activities of fraction V, from 40-50% to 10-20% of the activity applied to the gradient.

As the divalent cation concentration was reduced from (i)a to (i)b the activities of fractions IV and V decreased, while the activity of the pellet increased. A further reduction from (i)a to (i)c resulted in decreases in fractions I and II and a further increase in the pellet. When the divalent cation concentration was reduced from (ii)a to (ii)b, there were decreases in the activities of all of the fractions, except IV. No activity could be detected in fractions I or II of gradients (i)c or (ii)b.

The values given are the mean of 4-7 experiments performed in duplicate \pm the standard error of the mean. The gradients are labeled as in TABLE 1.1 and $p < 0.05$ by students T test for all of the alterations quoted above.

<u>Gradient fraction</u>	<u>(i)a</u>	<u>(i)b</u>
I	3.4 ± 0.4	3.4 ± 1.2
II	4.4 ± 0.2	4.5 ± 1.0
III	5.4 ± 1.2	3.8 ± 1.0
IV	10.2 ± 1.8	4.2 ± 1.4
V	54.2 ± 12.9	12.4 ± 1.4
Pellet	12.7 ± 3.0	23.3 ± 6.7

TABLE 1.4

<u>(i)c</u>	<u>(ii)a</u>	(ii)b
-	3.2 ± 0.4	-
-	9.3 ± 0.7	-
5.0 ± 0.5	7.3 ± 1.4	3.4 ± 0.5
7.7 ± 1.4	9.0 ± 1.9	13.7 ± 2.5
11.6 ± 2.0	47.3 ± 15.0	21.8 ± 3.8
32.0 ± 5.0	38.0 ± 9.0	16.7 ± 1.8

Potter Homogenisation. The gradient profile of cells disrupted by Potter homogenisation was not significantly different from that of syringe disrupted cells (Fig. 1.9). The relative specific activity of 5'-nucleotidase in fraction II of gradient I in the presence of 1mM $MgCl_2$ (6.6 ± 1.3 units) was not significantly different from that obtained after syringe homogenisation under these conditions and neither was the relative specific activity of NADH-ferricyanide reductase. It was concluded that syringe homogenisation was as effective as Potter homogenisation. However, the standard error is much larger in most of the fractions of the latter procedure indicating that its effect might not be so reproducible, possibly because it gives rise to a greater range of shearing stresses.

Gradient II. An attempt was made to determine whether an improvement in separation might be achieved by an alteration of the sucrose gradient. In the presence of 1mM $MgCl_2$ the cell homogenate was adjusted to 45% with respect to sucrose and layered on gradient II (see Methods). In this case (Fig. 1.10) there was a three fold increase in the relative specific activity of 5'-nucleotidase in fractions I, II and III. The values obtained were significantly less than those found in fraction II of gradient I. There were two peaks of rotenone insensitive NADH-ferricyanide reductase activity in gradient II, fractions I and V. The relative specific activity of this enzyme in fractions I, II and III was not significantly different in gradients I and II.

Fig. 1.9 The effect of Potter homogenisation on the gradient distribution of (a) 5'-nucleotidase and (b) rotenone insensitive NADH-ferricyanide reductase.

Potter homogenisation showed no significant advantage over syringe homogenisation. The highest relative specific activity for 5'-nucleotidase was found in fractions II and III (x6). The highest relative specific activity of rotenone insensitive NADH-ferricyanide reductase was also found in fraction III (x3.5). On the basis of these observations the best plasma membrane fraction was to be obtained from fraction II. However, the activity obtained from this fraction was less than that in the same fraction of gradient (ii)b when the cells had been syringe homogenised.

FIG. 1.9

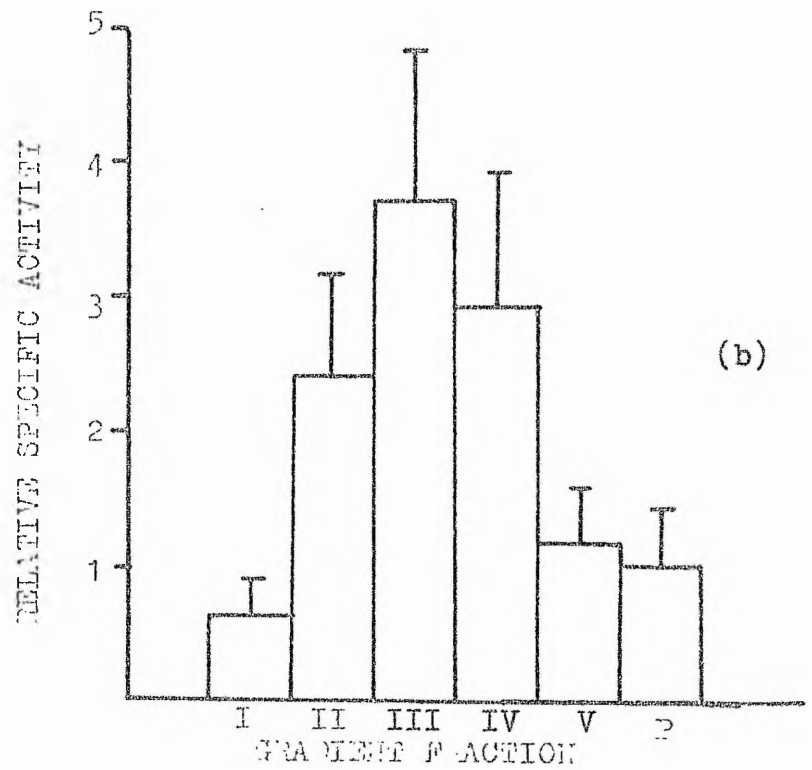
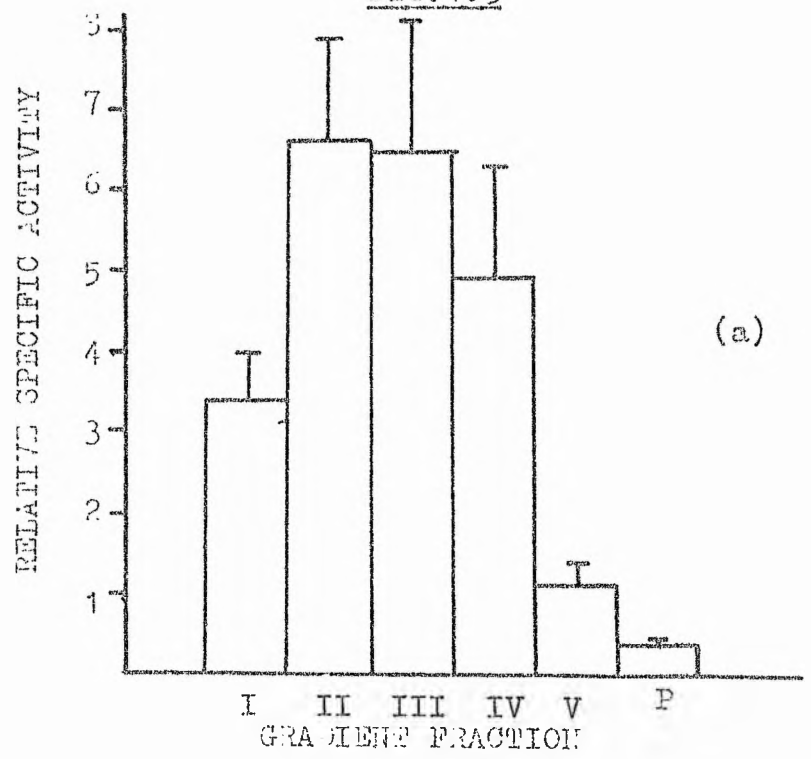


Fig. 1.10 The distribution of (a) 5'-nucleotidase and (b) rotenone insensitive NADH-ferricyanide reductase activity in the various fractions from gradient II.

The highest relative specific activity of 5'-nucleotidase (x3) was found in fractions I, II and III, while the highest relative specific activity of rotenone insensitive NADH-ferricyanide reductase (x2.5) was to be found in fraction I. The optimum plasma membrane isolation would, based on these criteria, be provided by fractions II and III. However, the relative specific activity of 5'-nucleotidase in these fractions was much less than was obtained in gradient I under the same divalent cation conditions.

The composition of the gradient is given in the methods section. The vertical columns represent the mean of 3 experiments performed in duplicate and the bars represent the standard error of the mean.

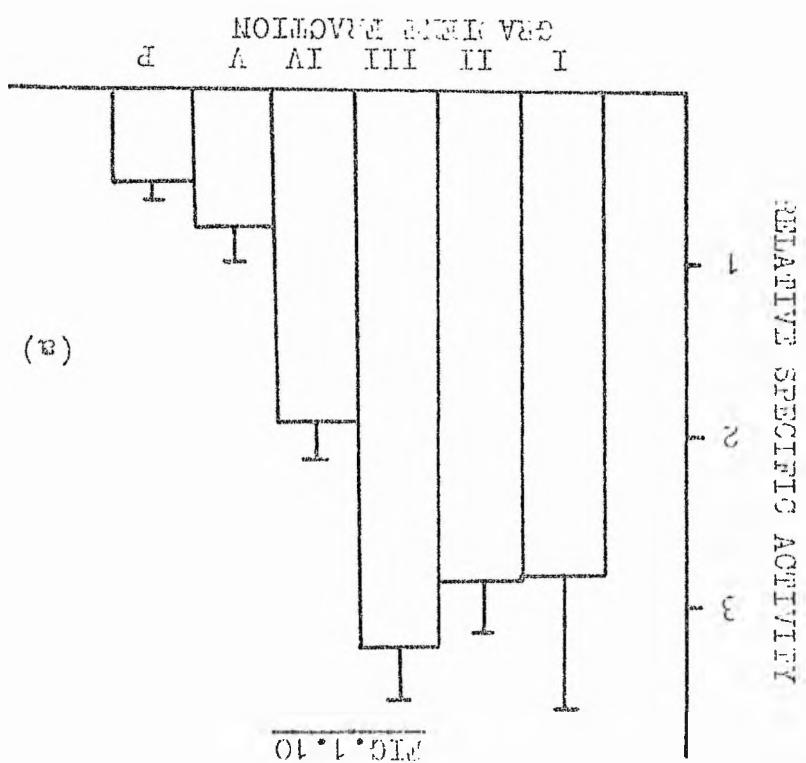
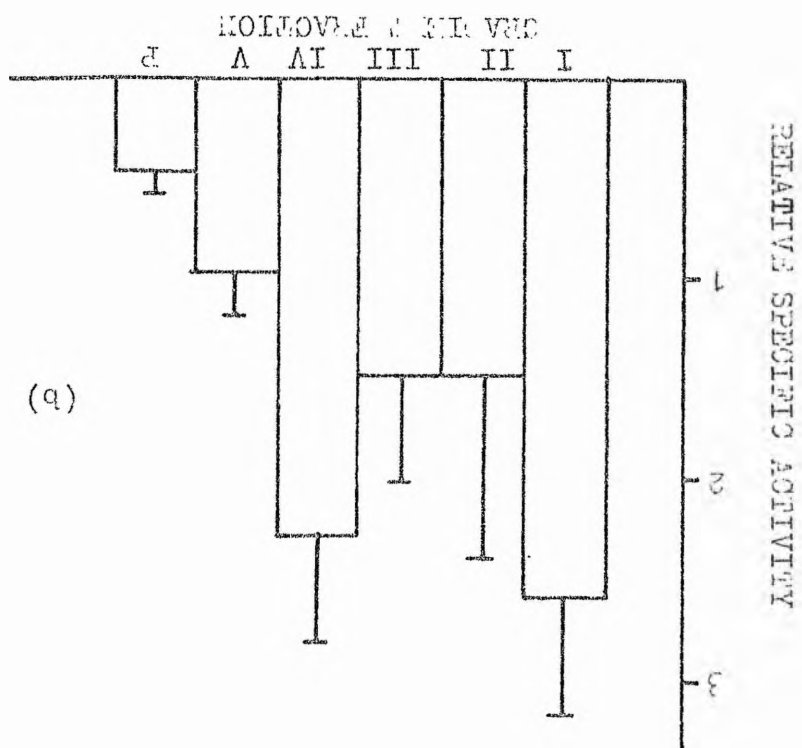


FIG. 1.10

(Na+K)-ATPase. Since the object of the isolation procedure was to provide a plasma membrane enriched in (Na+K)-ATPase, it would have been instructive, during these experiments, to compare the relative specific activity of this enzyme in the various gradient fractions. The activity of this enzyme, however, proved elusive. Different assay methods were attempted and fresh buffer and substrate were prepared to no avail. It was discovered, eventually, that the activity of the enzyme could be detected in the whole cell homogenate when the assay medium contained 0.1mM EDTA. However, no activity was detected in any of the gradient fractions unless 0.1mM EDTA was included throughout. The lack of activity was therefore attributed to heavy metal contamination.

The relative specific activity of 5'-nucleotidase and (Na+K)-ATPase from gradient I containing 1mM $MgCl_2$ + 0.1mM EDTA is shown in Fig. 1.11. The relative specific activity of 5'-nucleotidase (Fig. 1.11(a)) in fraction II was reduced to 5 fold in the presence of 0.1mM EDTA and the activity of the whole cell homogenate was reduced from 109 ± 13 to 28 ± 5 nM/mg/Hr. The highest relative specific activity of (Na+K)-ATPase was present in fraction V. Johnsen (1974) suggested that the activity of 5'-nucleotidase was better preserved in the presence of low concentrations of Ca^{++} . Calcium (0.5mM) was included throughout the procedure and, in order to retain a final divalent cation concentration of 1mM, the $MgCl_2$ was reduced to 0.5mM.

Isolation of (Na+K)-ATPase enriched material from SV 3T3 cells.

The specific activity and percentage recovery of several

Fig. 1.11 The specific activity of (a) 5'-nucleotidase and (b) (Na+K)-ATPase from SV40 3T3 cells in gradients containing 0.1mM EDTA and 1.0mM $MgCl_2$.

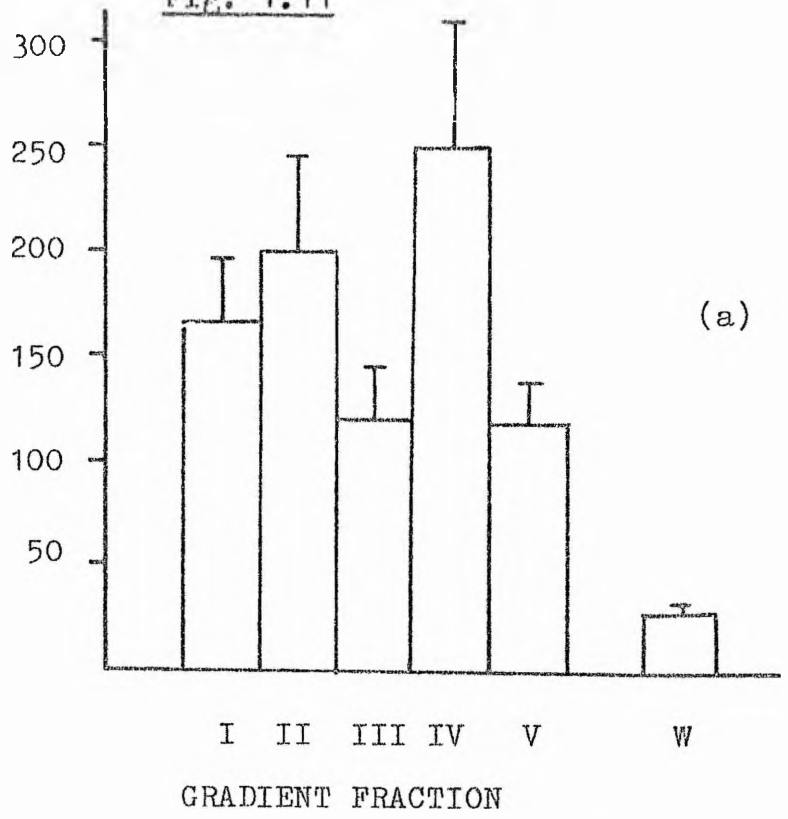
The highest specific activity of 5'-nucleotidase was obtained in fraction IV but the activity of the whole cell homogenate was markedly reduced in the presence of EDTA (from 109 ± 13 to 30 ± 5 nM/mg/Hr).

In none of the gradient fractions was the specific activity of (Na+K)-ATPase greater than that of the whole cell homogenate.

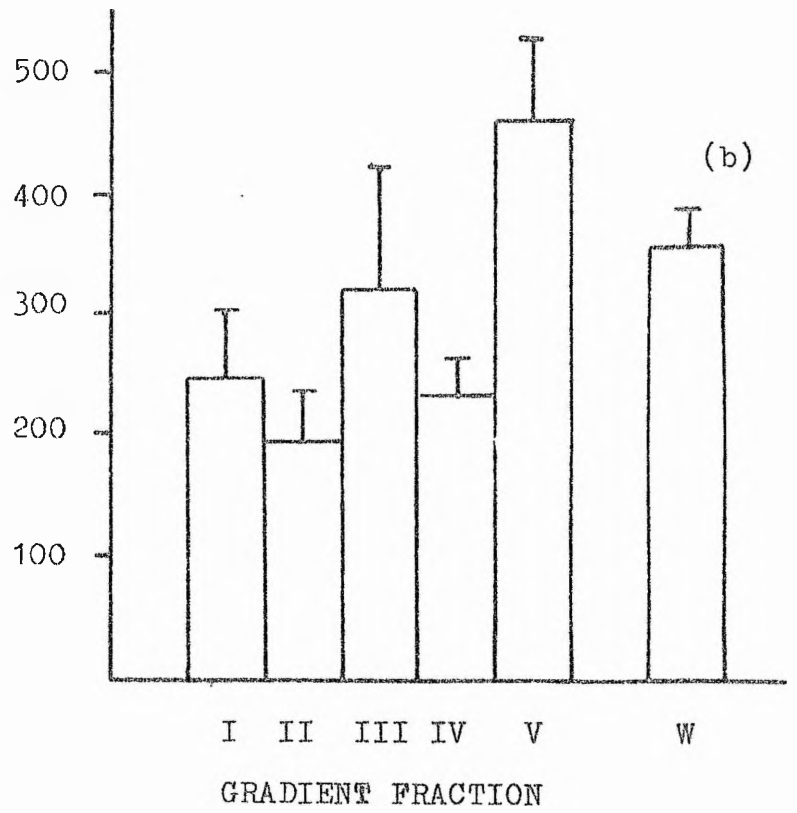
The columns represent the mean of 3 experiments performed in duplicate and the vertical bars represent the standard error of the mean.

Fig. 1.11

SPECIFIC ACTIVITY (cM/mg/Hr)



SPECIFIC ACTIVITY (cM/mg/Hr)



marker enzymes of SV 3T3 cells is shown in Table 1.5. The homogenised material was layered on gradient I containing 0.5mM $MgCl_2$ +0.5mM $CaCl_2$ +0.1mM EDTA, as a suspension of 40% with respect to sucrose and the various fractions were isolated as previously described.

The activity of glucose-6-phosphatase was below the limits of detection in all of the gradient fractions and this smooth E.R. marker is not included in the table.

The relative specific activity of 5'-nucleotidase was greatest in fraction IV (x6). The activity of the enzyme in this fraction was not, however, significantly different from that of fraction III. Fraction III had the greatest specific activity and percentage recovery of (Na+K)-ATPase. The total recovery of both of the plasma membrane markers was not significantly less than 100%.

The relative specific activities of rotenone insensitive NADH-ferricyanide reductase, succinate dehydrogenase and monoamine oxidase were highest in fraction IV and this fraction had the highest percentage recovery of these enzymes. The total recovery of monoamine oxidase was slightly greater than succinate dehydrogenase (43% c.f. 65%) and 69% of the total NADH-ferricyanide reductase was recovered. The activity of monoamine oxidase in the lower density fractions was less than that of succinate dehydrogenase. This observation is curious since it would be expected that a greater quantity of monoamine oxidase, the marker for the lower density outer membrane, would be found in the lighter fractions if the mitochondria were disrupted.

The total recovery of acid phosphatase was 43% and its

TABLE 1.5 The specific activity and percent recovery of various marker enzymes during the isolation of plasma membrane from SV40 3T3 cells.

Fraction III afforded the best plasma membrane preparation. In this fraction the relative specific activities of 5'-nucleotidase, (Na+K)-ATPase, rotenone insensitive NADH-ferricyanide reductase, acid phosphatase, succinate dehydrogenase and monoamine oxidase were 5.7, 10.7, 2.5, 2, 1 and 0.8 respectively. The highest relative specific activity of rotenone insensitive NADH-ferricyanide reductase(2.7) and monoamine oxidase(2.3) were recovered in fraction IV. The highest relative specific activity of acid phosphatase(2.5) was found in fractions I and II. Fraction II also had the highest relative specific activity of succinate dehydrogenase(2.8).

The values given are the mean of 5-10 experiments performed in duplicate \pm the standard error of the mean. (i): specific activity in * nM/mg/hr. or + μ M/mg/hr.; (ii): percent recovery.

TABLE 1.5

Enzyme	Whole cell	Gradient fraction					PELLET
		I	II	III	IV	V	
5'-Nucleotidase	(i)*	N.D.	153 \pm 32	588 \pm 64	620 \pm 61	120 \pm 12	53 \pm 32
	(ii)		2 \pm 0.5	35 \pm 5	30 \pm 3	10 \pm 1	7 \pm 4
(Na+K)-ATPase	(i)*	N.D.	720 \pm 80	1356 \pm 352	368 \pm 144	180 \pm 23	18 \pm 36
	(ii)		6 \pm 2	65 \pm 15	11 \pm 3	11 \pm 2	2 \pm 4
NADH-ferricyanide reductase	(i)+	114 \pm 75	120 \pm 72	263 \pm 69	294 \pm 35	189 \pm 54	129 \pm 36
	(ii)	3 \pm 1	2 \pm 1	14 \pm 4	13 \pm 4	18 \pm 4	19 \pm 6
Acid phosphatase	(i)*	1400 \pm 350	1400 \pm 350	1190 \pm 455	1120 \pm 385	259 \pm 46	136 \pm 24
	(ii)	7 \pm 3	4 \pm 1	11 \pm 3	10 \pm 3	5 \pm 3	3 \pm 1
Succinate dehydrogenase	(i)+	9 \pm 4	11 \pm 3	4 \pm 2	7 \pm 1	6 \pm 1	5 \pm 2
	(ii)	5 \pm 3	4 \pm 1	5 \pm 2	9 \pm 1	23 \pm 2	16 \pm 6
Monoamine oxidase	(i)+	0.4 \pm 0.1	0.6 \pm 0.2	0.6 \pm 0.1	1.9 \pm 0.4	1.5 \pm 0.4	0.7 \pm 0.2
	(ii)	0.8 \pm 0.2	1.9 \pm 0.6	3.0 \pm 0.5	9.0 \pm 1.0	20.0 \pm 7.0	8.0 \pm 2.0

highest relative specific activity occurred in fractions I and II. This observation is also curious since the buoyant density of the lysosomes is reputed to be 1.18-1.26 g/cc and the greater portion of this enzyme would be expected to occur in fractions IV and V.

The nuclear fraction contained no significant levels of (Na+K)-ATPase activity or 5'-nucleotidase activity but rather high levels of other marker enzymes.

A secondary purpose of these experiments was to provide purified nuclei in order to study the relationship between the nuclear/^{ENVELOPE}nucleoside triphosphatase (Agutter et al 1976) and nucleo-cytoplasmic translocation of ribonucleoprotein. This aim was achieved by increasing the sucrose loading density of the sample to 55%. The activities of some of the marker enzymes in nuclei and fraction III from gradients loaded with 40 and 55% sample material are compared in Table 1.6. The nuclear activity of 5'-nucleotidase and (Na+K)-ATPase in both cases was very small. In the latter case, however, the specific activity of acid phosphatase was reduced to 65% and NADH-ferricyanide reductase was reduced to 10% of the former. There was no detectable succinate dehydrogenase activity and the DNA recovery was increased from 77 ± 9 to $90 \pm 4\%$ when the sample was loaded as a 55% sucrose solution. In fraction III the recovery of 5'-nucleotidase, (Na+K)-ATPase and NADH-ferricyanide reductase is not significantly altered. However, the activity of acid phosphatase and succinate dehydrogenase was markedly higher (21 ± 8.4 c.f. $11.3 \pm 3\%$ and 12 ± 2.2 c.f. 5.2 ± 2.6 ; $p=0.3$ and <0.5 respectively.)

TABLE 1.6 A comparison of the pellet and fraction III from gradient I loaded with sample (a) 40%(w/w) with respect to sucrose or (b) 55% (w/w) with respect to sucrose.

In condition (a) the nuclear pellet was largely contaminated with lysosomes, endoplasmic reticulum and mitochondria. The extent of this contamination was greatly reduced if the sample was loaded onto the gradient as a solution 55% with respect to sucrose. The specific activities of acid phosphatase, rotenone insensitive NADH-ferricyanide reductase and succinate dehydrogenase in the pellet became reduced by 34%, 100% and 88% respectively. At the same time the DNA content of the pellet increased from 80-90% of that applied to the gradient.

When the sample was loaded as a suspension 55% (w/w) with respect to sucrose, fraction III became contaminated to a much greater extent by the activities of succinate dehydrogenase and acid phosphatase. At the same time the levels of (Na+K)-ATPase and rotenone insensitive NADH-ferricyanide reductase were reduced and the level of 5'-nucleotidase increased.

The values given are the mean of 3-7 experiments performed in duplicate \pm the standard error of the mean.

TABLE 1.6

<u>Enzyme</u>	<u>Fraction III</u>		<u>Nuclear Fraction</u>	
	<u>Specific activity</u>	<u>% recovery</u>	<u>Specific activity</u>	<u>% recovery</u>
5'-nucleotidase	(a) 588 \pm 64	18.4 \pm 6.0	53 \pm 32	6.5 \pm 4.1
	(b) 987 \pm 380	21.0 \pm 7.0	12 \pm 6.0	3.6 \pm 1.3
Na ⁺ /K ⁺ -ATPase	(a) 1856 \pm 352	65.0 \pm 15.0	Zero	Zero
	(b) 1240 \pm 450	17.5 \pm 6.1	Zero	Zero
NADH-ferricyanide reductase	(a) 264 \pm 69	14.2 \pm 3.0	129 \pm 36	19.3 \pm 5.8
	(b) 175 \pm 23	3.5 \pm 0.5	15 \pm 9	4.3 \pm 2.8
Succinate dehydrogenase	(a) 4 \pm 2	5.2 \pm 2.6	5 \pm 2.2	16.4 \pm 6.6
	(b) 22 \pm 4	12.0 \pm 2.2	Zero	Zero
Acid phosphatase	(a) 1190 \pm 455	11.3 \pm 3.0	136 \pm 24	3.4 \pm 0.6
	(b) 5440 \pm 2070	21.1 \pm 8.4	90 \pm 18	5.0 \pm 1.0

Plasma membrane from Py 3T3 cells. The method for the isolation of plasma membrane from SV3T3 cells proved applicable to Py3T3 cells without modification. Py3T3 cell homogenates (Table 1.7) possessed less 5'-nucleotidase and more (Na+K)-ATPase activity than SV3T3 cells. The activity of the other enzymes was not significantly different. Fraction III, as was the case with SV3T3 cells, possessed the highest specific activity and percentage recovery of both 5'-nucleotidase and (Na+K)-ATPase. The peak of rotenone insensitive NADH-ferricyanide reductase was obtained in fraction II. Succinate dehydrogenase activity was greatest in fractions I and V. Monoamine oxidase activity was distributed in a manner similar to that of succinate dehydrogenase. The highest specific activity of acid phosphatase was found in fraction III (x2). The major proportion of acid phosphatases, NADH-ferricyanide reductase, and succinate dehydrogenase was found in fraction V. The activities of 5'-nucleotidase and (Na+K)-ATPase in the nuclear fraction were low. The nuclei were, however, largely contaminated by succinate dehydrogenase, NADH-dehydrogenase and acid phosphatase.

Plasma Membrane from HeLa cells. Plasma membrane could also be prepared from HeLa cells by the method outlined above. In this case (Table 1.8) succinate dehydrogenase was below the limits of detection. The activities of 5'-nucleotidase and (Na+K)-ATPase in the whole cell homogenate were much greater than the 3T3 cell lines and the activities of NADH-ferricyanide reductase and acid phosphatase were much less. Fraction III, as was the case with the 3T3 cell lines, contained the

TABLE 1.7 The specific activity and percent recovery of various marker enzymes during the isolation of plasma membrane from Py 3T3 cells.

Fraction III provides the optimum plasma membrane preparation . This fraction contains 5'-nucleotidase, (Na+K)-ATPase, rotenone insensitive NADH-ferricyanide reductase, acid phosphatase, succinate dehydrogenase and monoamine oxidase in relative specific activities of 7.0, 14.0, 4.0, 2.3, 1.0 and 0.7 respectively. The highest relative specific activities of (Na+K)-ATPase and 5'-nucleotidase were found in this fraction. The highest relative specific activities of rotenone insensitive NADH-ferricyanide reductase, succinate dehydrogenase and monoamine oxidase were found in fractions I,II and I respectively.

The values given are the mean of 5-8 experiments performed in duplicate \pm the standard error of the mean. (i): specific activity in * nM/mg/Hr. or + μ M/mg/Hr.; (ii): percent recovery.

TABLE 1.7

<u>Enzyme</u>	<u>Whole cell</u>	<u>I</u>	<u>II</u>
5'-nucleotidase (i)*	74 \pm 7	438 \pm 123	424 \pm 186
		10 \pm 3	11 \pm 5
(Na+K)-ATPase (i)*	253 \pm 45	223 \pm 122	885 \pm 374
		2 \pm 1	7 \pm 3
NADH-ferricyanide (i)+ reductase (ii)	110 \pm 26	99 \pm 8	692 \pm 57
		1.5 \pm 0.1	12 \pm 1
Succinate (i)+ dehydrogenase (ii)	5 \pm 2	11 \pm 2	6 \pm 3
		4 \pm 1	2 \pm 1
Acid phosphatase (i)*	450 \pm 78	795 \pm 235	897 \pm 120
		3 \pm 1	4 \pm 0.5
Monoamine (i)+ oxidase (ii)	1 \pm 0.2	2 \pm 0.4	2 \pm 0.5
		3 \pm 1	3 \pm 1

Gradient fraction:

<u>III</u>	<u>IV</u>	<u>V</u>	<u>PELLET</u>
520 \pm 200	246 \pm 62	63 \pm 18	58 \pm 41
18 \pm 7	22 \pm 5	34 \pm 10	5 \pm 4
3625 \pm 959	1041 \pm 589	391 \pm 192	192 \pm 110
37 \pm 10	27 \pm 15	62 \pm 20	5 \pm 3
428 \pm 30	258 \pm 19	79 \pm 46	92 \pm 27
10 \pm 1	15 \pm 1	29 \pm 16	6 \pm 2
5 \pm 3	4 \pm 1	11 \pm 1	3 \pm 1
3 \pm 2	5 \pm 1	88 \pm 8	4 \pm 1
1020 \pm 123	493 \pm 45	582 \pm 202	285 \pm 134
6 \pm 1	7 \pm 1	52 \pm 18	4 \pm 2
1 \pm 0.2	1.5 \pm 0.3	2 \pm 0.4	0.3 \pm 0.1
2 \pm 0.5	8 \pm 2	68 \pm 16	2 \pm 1

TABLE 1.8 The specific activity and percent recovery of various marker enzymes during the isolation of plasma membrane from HeLa cells.

As was the case with SV40 and Py 3T3 cells, the highest relative specific activities of the plasma membrane markers were found in fraction III. In this fraction the relative specific activities of 5'-nucleotidase, (Na+K)-ATPase, rotenone insensitive NADH-ferricyanide reductase, acid phosphatase and monoamine oxidase were 5.7, 5.4, 1.0, 1.5 and 0 respectively. The highest relative specific activities of rotenone insensitive NADH-ferricyanide reductase(1.6) and monoamine oxidase(3.9) occurred in fractions II and V respectively. No succinate dehydrogenase activity could be found in the whole cell homogenate or any of the gradient fractions.

The values shown are the mean of 4-7 experiments performed in duplicate \pm the standard error of the mean. (i): specific activity in * nM/mg/Hr. or + μ M/mg/Hr. (ii): percentage activity recovered.

TABLE 1.8

Enzyme		Whole cell	Gradient fraction					Pellet
			I	II	III	IV	V	
5'-nucleotidase	(i)*	361 \pm 26	979 \pm 130	1918 \pm 138	2056 \pm 100	828 \pm 276	301 \pm 44	zero
	(ii)		5 \pm 1	12 \pm 1	28 \pm 1	23 \pm 8	16 \pm 2	
Na ⁺ /K ⁺ -ATPase	(i)*	317 \pm 70	277 \pm 50	814 \pm 240	1708 \pm 220	676 \pm 145	517 \pm 102	zero
	(ii)		2 \pm 0.5	6 \pm 2	27 \pm 3	21 \pm 4	32 \pm 6	
NADH-ferricyanide	(i)+	84 \pm 8	30 \pm 1	138 \pm 30	81 \pm 10	84 \pm 25	92 \pm 9	108 \pm 24
	(ii)		1 \pm 0.1	4 \pm 1	2 \pm 0.5	10 \pm 3	21 \pm 2	9 \pm 2
Acid phosphatase	(i)*	35 \pm 6	46 \pm 7	53 \pm 10	57 \pm 8	30 \pm 6	18 \pm 4	15 \pm 3
	(ii)		2 \pm 0.5	4 \pm 1	8 \pm 1	9 \pm 2	10 \pm 2	3 \pm 1
Monoamine oxidase	(i)+	0.7 \pm 0.1	1.0 \pm 0.33	N.D	N.D	1.3 \pm 0.2	2.7 \pm 0.4	0.9 \pm 0.1
	(ii)		4.0 \pm 0.8			12.0 \pm 2.0	48.0 \pm 7.0	6.0 \pm 2.0

highest specific activity of 5'-nucleotidase and (Na+K)-ATPase (x 5.4 and 5.7 respectively). In this fraction no monoamine oxidase activity could be detected and the relative specific activities of acid phosphatase and NADH-ferricyanide reductase were 1.6 and 1.1 respectively. Monoamine oxidase activity was greatest in fraction V and NADH-ferricyanide reductase activity was highest in fraction I.

DISCUSSION

Fraction III from gradients of HeLa, Py3T3 and SV40 3T3 cells prepared in 0.1mM EDTA + 0.5mM CaCl_2 + 0.5mM MgCl_2 are enriched in both 5'-nucleotidase and (Na+K)-ATPase. Since these enzymes are generally considered to be constituents of the plasma membrane, it follows that fraction III from these gradients is enriched in plasma membrane material. However, this conclusion is only valid if these enzymes act as plasma membrane markers in the systems described. That some proportion of the activity ascribed to these enzymes is located in the plasma membrane, is beyond question; but do these enzymes exist in other cell fractions and could the enrichment observed be due to the purification of some organelle other than the plasma membrane?

The most convincing evidence for the presence of 5'-nucleotidase in the plasma membrane is the ability to detect the enzyme in intact cells. In some cell types there appears to be an exo-5'-nucleotidase activity (Trams and Lauter 1974). In other cells the active site appears to be found on the inner surface of the plasma membrane (Williamson et al 1976). In these cells right side out plasma membrane vesicles contain one third of the activity of inside out vesicles. However, the activity of the 5'-nucleotidase has been shown by histochemistry to occur on other cell fractions (Widnell 1972). Unfortunately, histochemical treatment often leads to staining artifacts and even if this were not the case there would always be the problem of membrane

biosynthesis to consider (see Introduction).

In the mouse cells used in the present investigation 30±3% of the total cell 5'-nucleotidase may be observed on the cell surface. When the cells were disrupted by repeated syringing the remainder of the activity was liberated.

The activity of the (Na+K)-ATPase in the plasma membrane is not so easily demonstrated since its active site is reputed to be at the interior surface of the membrane. However, its presence in the plasma membrane is accepted by virtue of its undoubted association with the sodium pump. If both 5'-nucleotidase and (Na+K)-ATPase are exclusively located in the plasma membrane and neither are inactivated to any great extent during isolation, they should show the same increase in purity in the plasma membrane fraction. However, though most of the activity of both of these enzymes is recovered from the gradient, they do not show the same distribution throughout the gradients from SV40 and Py 3T3 cells. In gradients of SV40 3T3 cells the highest specific activity of 5'-nucleotidase was to be found in fraction IV, while the highest specific activity of the (Na+K)-ATPase was to be found in fraction III; whereas in Py 3T3 cells 38% of the (Na+K)-ATPase activity was found in fraction III which contained only 15% of the 5'-nucleotidase activity; fraction I contained 10% of the 5'-nucleotidase activity and only 0.5% of the (Na+K)-ATPase activity. In HeLa cell gradients the distribution of the two enzymes is not significantly different. However, the relative specific activity of fraction III is much less than that of the corresponding fractions from gradients of Py and SV40 3T3 cells.

According to the table devised by Neville (1976) vesiculated plasma membrane has a buoyant density in sucrose of 1.19-1.07 g/cc. The table on page 26 gives the composition of the gradient in terms of sucrose density and from the above data, material of that density would be expected to be a constituent of fractions I-V. In the three cell lines examined, the highest percentage recovery and the highest relative specific activity of both marker enzymes was found in fraction III, indicating that the plasma membrane from these sources had a density range of 1.12-1.15g/cc. The endoplasmic reticulum with a buoyant density of 1.06-1.23 g/cc. should also be found in gradient fractions I-V. Rotenone insensitive-NADH ferricyanide reductase, the E.R. marker used in these studies, occurs in fraction V to a greater extent than in any of the other gradient fractions of all 3 cell lines. However, the overall distribution of rotenone insensitive NADH-ferricyanide reductase depends on the cell type. In gradients of SV40 3T3 cells the highest relative specific activity of this enzyme occurred in fractions III and IV, in Py 3T3 cell gradients the highest relative specific activity was to be found in fractions II and III, while in HeLa cell gradients there are two peaks of activity in fractions II and V. It has been proposed that this enzyme is a component of the endoplasmic reticulum (de Pierre and Dallner 1976). However, Zamudio (1969) has observed its activity in the erythrocyte ghost and Berezney et al (1970) have shown that it also occurs in the nuclear envelope. Whether or not this enzyme is a constituent of the plasma membrane or the E.R. or both, its distribution in the

gradient will depend on the size of the vesicles which these structures form. This in turn will probably vary with the cell type. Apart from intrinsic differences, each cell type requires a different number of passages to disrupt it and this in itself will probably produce different vesicle sizes.

The lysosomes with their buoyant density of 1.19-1.22 g/cc. should be found in fraction V. Acid phosphatase, the enzyme used to monitor the distribution of these organelles, was found to a greater extent in fraction V than in any of the other fractions of gradients from HeLa and Py 3T3 cells. In SV40 3T3 cell gradients the highest percentage recovery was found in fraction III. The reason for this is not certain, but may be attributable to the proposed activity of p-nitrophenyl phosphatase in the plasma membrane.

Acid phosphatase has been widely used as a marker from the lysosomal component of the cell (Wattiaux 1977). Its activity was estimated in these experiments as the rate of hydrolysis of p-nitrophenyl phosphate at pH 6.5. However, it has been reported recently that there are several isoenzymes of acid phosphatase in 3T3 cells (Beadle et al 1976). The isoenzymes have different substrate specificities such that the plasma membrane isoenzyme preferentially hydrolyses p-nitrophenyl phosphate and the lysosomal component preferentially hydrolyses β -glycerophosphate. Unfortunately, time did not permit the estimation of the activity of β -glycerophosphatase. In all three cell lines the highest relative specific activity of p-nitrophenyl phosphatase was found in fraction III. This observation tends to confirm the results of Beadle et al. and suggests that there may be

a plasma membrane component of the cell acid phosphatase activity.

When homogenates of SV40 3T3 cells are treated with deoxycholate there is only a 10% increase in activity. Since lysosomal hydrolases are generally considered to be cryptic this observation tends to suggest that either disruption of the lysosomes occurs on cell homogenisation or that there is a non lysosomal component of acid phosphatase activity. This observation ~~also~~ tends to suggest that there may be a non-lysosomal component of acid phosphatase activity. The recoveries of acid phosphatase activity from the gradients are very low (around 20-50%). It is, therefore, not possible to compare the distribution of the enzymes in gradients from different sources. Except for SV 3T3 cell gradients, the bulk of the activity of this enzyme is located in gradient fractions of greater density than the plasma membrane fraction. In gradients from SV40 3T3 cells the highest percentage recovery occurred in fractions III and IV, in Py 3T3 cell gradients the highest percentage recovery was obtained from fraction V and fractions IV and V of HeLa cell gradients contained the bulk of the activity.

From their buoyant density values (1.17-1.21 g/cc) the mitochondria should occur predominantly in fractions IV and V. Indeed, in all cases, the highest percentage recovery of both monoamine oxidase and succinate dehydrogenase

was found in fraction V. These enzymes are considered markers for the inner and outer mitochondrial membranes respectively. Since the outer membrane has the lower density, the activity of monoamine oxidase in the lower density fractions would be expected to be higher than that of succinate dehydrogenase in the case of fragmentation of the mitochondria. The activities of these enzymes in SV40 and Py 3T3 cell homogenates are not significantly different. The slightly asymmetric distribution of monoamine oxidase and succinate dehydrogenase in gradients of Py and SV40 3T3 cells is thought to reflect the relative recoveries of these enzymes. In gradients of SV40 3T3 cell homogenates there is a greater percentage recovery of succinate dehydrogenase than of monoamine oxidase and in each of the gradient fractions except III, IV and V the succinate dehydrogenase activity is greater than that of monoamine oxidase. In gradients of Py 3T3 cell homogenates the recovery of monoamine oxidase is greater than that of succinate dehydrogenase and in fractions II, IV and pellet the relative specific activity of monoamine oxidase is greater than that of succinate dehydrogenase.

Succinate dehydrogenase activity could not be detected in HeLa cell homogenates or gradient fractions. The monoamine oxidase activity of HeLa cell homogenates was not significantly different from that of Py or SV 3T3 cells and the total recovery of enzyme from the gradient was approximately the same. However, no monoamine oxidase activity could be detected in fractions II or III of HeLa cell gradients. The highest relative specific activity

and percent recovery of this enzyme was obtained from fraction V of HeLa cell gradients.

The nuclear material (buoyant density greater than 1.3 g/cc.) was expected to form a pellet at the base of the gradient. This in fact was the case. Indeed, with proper manipulation of the gradient, the nuclear material was pure enough to use for the preparation of nuclear envelope. The modified procedure was used to produce nuclear envelope for the investigation of some of the properties of the nuclear envelope nucleoside triphosphatase (Agutter et al 1976).

Homogenisation. Cells may be homogenised by several methods (see Introduction). Syringe homogenisation was chosen, in this instance, because it seemed controllable and reproducible. The results obtained from syringe homogenised material compared favourably with those obtained from Potter homogenised material. However, the choice of the syringe needle is important. Needles of internal diameter 0.41mm produced optimum homogenisation; larger diameter needles disrupted the cells very slowly, while needles of smaller diameter brought about the disruption of the nuclei also.

Divalent cations. Divalent cations are necessary constituents of isolation media in order to stabilise the nuclear material. However, the divalent cation regime must be rigidly controlled since it may affect the recovery and distribution of several marker enzymes.

The specific activity of 5'-nucleotidase in the low

density material tends to increase as the divalent cation concentration is reduced (Fig. 1.6). This is in agreement with the observations of Bingham and Burke (1972). However, it would appear that the increase, in this instance, is due not to an increase in the content of 5'-nucleotidase, but to a decrease in the protein content of the lower density gradient fractions (Tables 1.2 and 1.1).

In contrast to 5'-nucleotidase the total recoveries of both rotenone insensitive NADH-ferricyanide reductase and succinate dehydrogenase (Tables 1.3 and 1.4) are affected by reductions in the divalent cation concentration. As the divalent cation concentration is reduced below 2.0mM the recovery of both of these enzymes is sharply reduced and approximately 50% of the total activity is lost. This decrease is paralleled by a reduction in the total protein recovered from the gradient. The loss of enzyme activity and protein is greatest from fraction V.

The bimodal distribution of succinate dehydrogenase in the presence of Ca^{++} has already been discussed. In the presence of 0.5mM Ca^{++} there are two peaks of activity, a low and a high density peak. It appears that Ca^{++} may bring about the disruption of the mitochondria and this would be consistent with the reported detrimental effect of Ca^{++} on mitochondrial activity (Scottocasa 1976).

The highest relative specific activity of 5'-nucleotidase was usually obtained from fractions II or III of gradients depending on the divalent cation concentration. In the presence of 1mM MgCl_2 fraction II contained the highest relative specific activity found in any of the gradients.

EDTA. The inclusion of EDTA proved necessary in order to retain the activity of the (Na+K)-ATPase. During the preliminary experiments on the effect of divalent cation concentration on the distribution of 5'-nucleotidase activity, the best purification was found in fraction II of gradients containing 1mM MgCl₂. However, in the presence of 1mM MgCl₂ alone, no (Na+K)-ATPase activity could be detected in the homogenate or any of the gradient fractions. In the absence of EDTA the activity of the (Na+K)-ATPase proved elusive. In the presence of 1mM MgCl₂ and 0.1mM EDTA the activity of the 5'-~~nuc~~leotidase in both the whole cell homogenate and the gradient fractions was greatly reduced (Fig. 1.11). The activity of 5'-nucleotidase could, however, be stabilised by the addition of Ca⁺⁺ to the isolation buffer. These observations tend to suggest that traces of Ca⁺⁺ in the buffer normally stabilise or protect the activity of the 5'-nucleotidase; when these are chelated by EDTA, the activity of the enzyme is lost. The activity of the (Na+K)-ATPase is apparently inhibited by trace heavy metal ions which are removed in the presence of EDTA.

These observations are in agreement with those of Johnsen et al who showed that the 5'-nucleotidase of HeLa cells was inhibited in the presence of EDTA and that the activity could be preserved by the inclusion of Ca⁺⁺ in the isolation procedure. These authors also showed that 0.1mM EDTA was necessary in order to preserve the activity of the (Na+K)-ATPase. These isolation conditions may be used to prepare plasma membrane from SV and Py 3T3 and HeLa cells in gradient I (Tables 1.5, 1.8 and 1.9).

The plasma membrane preparation compares favourably with several of the methods reported in the literature. The points to be considered are 1) the activity of 5'-nucleotidase with respect to the whole cell, the relative specific activity and 2) the operation time. The times given below are the centrifugation times and the operation times will usually be 2-3 hours greater than these.

Operation time is important since, if this is prolonged, spuriously high estimates of purity might be obtained. Contamination is often monitored by the estimation of the activity of various enzymic markers, if these are inhibited during long preparation times the estimated contamination will be lower than the actual contamination.

Several methods considered here have prolonged operation times; these include the methods of Touster et al (1970), Johnsen et al (1974) and Bosmann et al (1968). Each of these methods require over 12 hrs. for completion.

The method of Touster et al, developed for liver cells, involves a total centrifugation time of 18 hr. and produces material of buoyant density 1.14 g/cc which is 20-30 fold enriched in 5'-nucleotidase and phosphodiesterase activities. They estimated that some 20% of the material was due to E.R. and that 2% was of mitochondrial origin. Preliminary attempts at this method yielded a fraction from HeLa cells which was enriched 4 fold in 5'-nucleotidase activity but contained only 10% of the total cell activity of this enzyme.

Johnsen et al (1974) published a method for the isolation of plasma membrane from HeLa cells which took some 18 hrs. The material, which was enriched 7 fold in 5'-nucleotidase activity and 19 fold in (Na+K)-ATPase activity, had a buoyant density of 1.16 in the presence of EDTA and 1.20 in its absence. It was suggested that 5'-nucleotidase was not a good plasma membrane marker since its activity did not closely follow that of the (Na+K)-ATPase. The disparity between these results and the present data may be due to a difference between the spinner cells used by Johnsen et al and the 'substratum' cultures used in the present study, or it may be due to methodological differences. Johnsen assayed for 5'-nucleotidase at pH 8.5 which is much higher than the 7.4 reported optimum for the enzyme. At the higher pH they would probably detect hydrolysis of the AMP by alkaline phosphatases, which are widely distributed in the cell.

Bosman et al (1968), using a procedure of 32 hr. duration isolated plasma membrane from HeLa cells which was reputed to be 120 fold enriched in 5'-nucleotidase activity and 30 fold enriched in the activity of the (Na+K)-ATPase. The enrichment of the 5'-nucleotidase, while theoretically possible, is unlikely in practice. As Johnsen et al (1974) observed, the activity of 5'-nucleotidase is suppressed in the presence of EDTA, so that the results of Bosman et al might be explained if they had included EDTA in the homogenisation buffer but had omitted it from the gradient. Shorter procedures have been published by Atkinson (1973), Bingham and Burke (1972), Dorling and LePage (1973),

Lelievre (1973), Nigam et al (1971) and Forte et al (1973). These methods require 3-5 hrs. preparation time and yield material 4-15 fold increased in the relative specific activity of plasma membrane markers.

Atkinson (1973) in his method of isolation of plasma membrane from HeLa cells includes no data on enzyme activity. The method involved 5 hrs. centrifugation time. Preliminary attempts at the preparation of plasma membrane from HeLa cells by this method produced material 2 fold enriched in 5'-nucleotidase and which had half of the specific activity of rotenone insensitive NADH ferricyanide reductase of the whole cell fraction.

Bingham and Burke (1972) reported an isolation procedure for the preparation of plasma membrane from chick embryo fibroblasts which also involved 5 hrs. centrifugation time. The cell surface material was enriched 5 fold in 5'-nucleotidase activity in 0.5mM $MgCl_2$ and 0.5 fold in 5mM $MgCl_2$.

The method of Dorling and LePage (1973) has a 4 hr. centrifugation time and provides material which is enriched 25 fold in 5'-nucleotidase activity.

LeLievre (1973) prepared plasma membrane from murine plasmocytoma cells in 3 hrs. The membrane material had a 3 fold increase in the specific activity of 5'-nucleotidase and a 4-7 fold increase in the specific activity of the (Na+K)-ATPase. The method of Nigam et al (1971) also involved 3 hrs. centrifugation time. This method produced, from rat liver, material which had a density of 1.16-1.18 g/cc² and was 12 fold enriched in 5'-nucleotidase activity.

Forte et al (1973) have prepared plasma membrane material

from Ehrlich ascites tumour cells. The relative specific activity of (Na+K)-ATPase was 15 and no data was included for 5'-nucleotidase.

In conclusion, it would seem that fraction III, which contains a large proportion of the plasma membrane markers, may also contain acid phosphatase activity as well as traces of rotenone insensitive NADH-ferricyanide reductase, succinate dehydrogenase and monoamine oxidase activity. Acid phosphatase and rotenone insensitive NADH-ferricyanide reductase may be components of the plasma membrane but the presence of the other two enzymes was not expected. However, the latter are not enriched in fraction III from gradients of any cell line.

The (Na+K)-ATPase activity of the cultured cells investigated is enriched 6-15 times in fraction III and from 27-65% of the total activity of the enzyme is recovered in this fraction.

The method outlined in the above text is, therefore, superior to previously published methods in respect of operation time and compares favourably in respect of purification of the plasma membrane marker enzymes. Therefore, this method might be profitably employed in the preparation of plasma membrane material for the study of some of the properties of the plasma membrane (Na+K)-ATPase.

CHAPTER II

CHARACTERIZATION OF SOME OF THE PROPERTIES OF THE (Na+K)-ATPase FROM SV40 3T3 AND 3T3 CELLS

SUMMARY

1. Plasma membrane was prepared from SV40 3T3 and 3T3 cells as outlined in Chapter I and this material was used to establish some of the properties of the (Na+K)-ATPase in these cells.
2. When the Na^+ concentration was maintained at 60mM, maximal stimulation of both enzymes was obtained in the presence of 10mM K^+ .
3. When the K^+ concentration was maintained at 10mM, maximal stimulation of both enzymes was obtained in the presence of 60mM Na^+ .
4. Using the optimum Na^+ and K^+ concentrations the rate of hydrolysis of ATP was linear for at least 30 min. with a MgATP^{2-} concentration of 0.8mM.
5. The K_i for ouabain inhibition of the (Na+K)-ATPase from SV40 3T3 cells was 0.07mM compared to 0.04mM in 3T3 cells.
6. Various adenine nucleotides also affected the rate of reaction. The 3T3 cell activity was more sensitive to 3'5' cyclic AMP and the SV40 3T3 cell activity was more sensitive to adenosine. However, both enzymes were unaffected by AMP and inhibited to the same extent by ADP.

7. The apparent K_m for $MgATP^{2-}$ was 0.07 ± 0.003 in the case of SV40 3T3 cells and 0.12 ± 0.01 in the case of 3T3 cells. The V_{max} of the enzyme was observed to be 1607 ± 30 in the case of SV40 3T3 cells and 1960 ± 30 in the case of 3T3 cells.
8. The effectiveness, as substrates, of various divalent cation-ATP complexes were examined. In both cases the activity decreased in the following way $Mg^{2+} > Mn^{2+} > Ca^{2+} > Be^{2+} > Ba^{2+} \simeq Zn^{2+}$.

INTRODUCTION

The sodium and potassium stimulated magnesium dependent ATPase, abbreviated to (Na+K)-ATPase, was discovered by Skou (1957) when he showed that the rate of hydrolysis of ATP by minced crab nerve was stimulated in the presence of both sodium and potassium ions. The hypothesis of the involvement of the (Na+K)-ATPase in the functioning of the sodium pump was, until quite recently, founded on circumstantial evidence based on the large number of similarities in the two systems. Both systems require identical optimum concentrations of Na^+ , K^+ , Mg^{++} and ATP (Whittam and Ager 1964), and are inhibited by extra-cellularly applied cardiac glycosides. In both cases there is the same relationship between the structure of the glycoside and its potency (Abeles 1969). Both systems have the same ionic selectivity (Baker 1965, 1972), and require the same asymmetric distribution of ions across the membrane; Na^+ and ATP at the interior and K^+ at the exterior surface (Perrone and Blostein 1973; Whittam 1962 and Whittam and Ager 1964). More direct evidence has been provided by studies on black lipid membranes. When a (Na+K)-ATPase 'of 95% purity' was introduced into a black lipid membrane a system was produced which was capable, on the hydrolysis of ATP, of the ouabain sensitive exchange of Na^+ and K^+ in the ratio of 2.8 to 2.0 (Hilden and Hokin 1975, Sweadner and Goldin 1975).

The enzyme is lipid dependent and the removal of lipid by detergent (Tanaka and Abbod 1964), phospholipase (Roelofsen et al 1966) and organic solvents (Jarnefelt 1972)

leads to partial or complete loss of activity. When the lipid component of the membrane is altered experimentally, the properties of the enzyme alter. The (Na+K)-ATPase of Ehrlich ascites cells from mice fed different fatty acid diets possessed different Arrhenius plot transition temperatures (Solomonson et al 1976).

The reactivation of the partially delipidated enzyme may be achieved by the addition of phospholipid. The lipid specificity appears to be dependent on the nature of the enzyme. Those preparations (Priestland and Whittam 1972, Tanaka et al 1971) in which only a small proportion of the lipid remains attached are reactivated to a greater extent by phosphatidyl serine than any other negatively charged or neutral phospholipid (Fenster and Copenhaver 1967; Kimelberg and Papahadjopoulos 1974; Wheeler and Whittam 1970). However, the treatment of ox brain plasma membrane with phosphatidyl serine decarboxylase, an enzyme which converts phosphatidyl serine to phosphatidyl ethanolamine, did not have a significant effect on the rate of hydrolysis of ATP (De Pont et al 1973). While this result tends to suggest that in situ phosphatidyl serine is not necessary for the function of the enzyme, the conclusion is equivocal since 5% of the total phosphatidyl serine remains unaltered and it is possible that this small fraction is responsible for maintenance of the activity of the enzyme. Those preparations in which most of the endogenous lipid remained attached to the enzyme had far less specificity for reactivation (Hokin and Hexum 1972). The enzyme isolated from dogfish rectal

gland, claimed to have a purity of greater than 95%, is associated with lipid in a molar ratio of 1:120 (Perrone et al 1975). The lipid component seems to be arranged in a bilayer which, according to ESR measurements, is more ordered in the vicinity of the protein (Grisham and Barnett 1972).

The protein component of the enzyme seems to show remarkable similarities in many of the preparations investigated. It appears to consist of two subunits: a) a large subunit with an apparent molecular weight in SDS polyacrylamide gel electrophoresis of approximately 90,000 and b) a small Mw subunit with an apparent molecular weight of 53,000. The large subunit becomes phosphorylated in the presence of Na^+ , Mg^{++} and ATP (Hokin 1974; Kyte 1971; Perrone et al 1975). The small subunit is a glycoprotein, the carbohydrate component of which depends on the source of the enzyme (Perrone et al 1975; Hokin 1974; Kyte 1972). There is a lack of direct evidence that the small subunit is intimately involved in the activity of the enzyme. However, the two components copurify and can be crosslinked in a 1:1 fashion by dimethyl suberimidate treatment of the intact membrane (Kyte 1972); the enzymic activity is inhibited by antibodies to the small subunit and by Concanavalin A binding (Rhee and Hokin 1975). The small subunit appears to function as the ionic carrier. Shammo and Albers (1973) reported the first evidence that the ion transport site may be a component of the (Na+K)-ATPase molecule. Cyanogen bromide digestion of the lubrol solubilised enzyme resulted in a fraction which had a K^+

dependent iontophoretic activity (Shamoo and Ryan 1975).

Due to the uncertainty involved in the estimation of protein molecular weight there is some dispute over the molar ratios of the two components of the sodium pump. Hokin (1974) has proposed that in dogfish rectal gland and in the electric organ of the eel, the large:small subunit ratio is 2:1, Jorgensen (1974) has suggested the same ratio for the electric organ of the eel, while Kyte (1972) has proposed that in rabbit kidney outer medulla the ratio of large:small subunits is 1:2. Stein et al, on the other hand (1973) have proposed that the pump is a dimer, each monomer unit of which consists of one large and one small subunit.

The sodium pump, it has been shown, may function in several modes. Glynn and Karlish (1975) have described 5 modes of operation: 1) $\text{Na}^+ - \text{K}^+$ exchange, the normal mode which requires internal ATP and Na^+ and external K^+ ; 2) the reverse mode which operates in the presence of low internal Na^+ , high internal K^+ and high external Na^+ ; 3) a $\text{Na}^+ - \text{Na}^+$ exchange mode which occurs in the presence of high external Na^+ and low external K^+ ; 4) a $\text{K}^+ - \text{K}^+$ exchange mode which functions in the absence of extracellular Na^+ , and 5) a Na^+ extrusion mode which occurs in the absence of external Na^+ or K^+ . There are therefore, both internal and external ion binding sites which have different ionic affinities. When a cell is disrupted, there will be no distinction between these sites and studies on the effect of ions on the enzyme activity will be non-physiological. The stimulation of the enzyme by Na^+ and K^+ varies from tissue to tissue and from preparation to preparation. The ion concentration for half

maximal stimulation of the enzyme varies from 5-13mM Na⁺ and 0.3-1.8mM K⁺ (Glynn and Karlsh 1975). The enzymic activity may or may not involve several steps which can be considered independently. The work of Albers (1973) and Post (1973) and others suggests that, during the hydrolysis of ATP the γ -phosphate group is transferred to a membrane protein to form an unstable complex. The large subunit, in the presence of Na⁺ and Mg⁺⁺, acts as a phosphate acceptor. This reaction does not require K⁺ and is not inhibited by ouabain. Post and Kume (1973) isolated a tripeptide in which the phosphate group appeared to be attached to an aspartyl beta carboxyl group. This observation was later substantiated by Nishigaki et al (1974) who showed that after reduction with ³H borohydride the phosphate was attached to ³H homoserine. Dephosphorylation requires the presence of K⁺ and is inhibited by ouabain. This step may be measured separately as a ouabain sensitive K⁺ stimulated p-nitrophenylphosphatase.

The pH optimum for the enzyme varies from 7.0 to 7.8 depending on the tissue and species of origin. The products of the reaction appear to inhibit, ADP competitively and Pi noncompetitively (Glynn and Karlsh 1975). The enzyme is also inhibited in the presence of cardiac glycosides such as ouabain and the concentration for half maximal inhibition (K_i) varies from 10⁻⁷M to 10⁻⁴M. The effect of ouabain is often biphasic; when this is the case there is an increase in the activity of the enzyme at concentrations around K_i/300 (Bonting 1970).

The (Na⁺K)-ATPase is usually associated with a MgATPase

activity (Bonting 1970) and a differential assay must be used for its estimation. The practice of comparing the rate of hydrolysis of ATP in the presence and absence of Na^+ and K^+ is liable to error since, in many tissues MgATPase is activated to a certain extent by Na^+ alone (Bonting 1970). The addition of K^+ and Na^+ to the assay medium induces the formation of KATP^{3-} and NaATP^{3-} , which effectively reduces the MgATP^{2-} concentration. On the other hand, the fact that ouabain insensitive fluxes may be associated with the pump (Glynn and Karlsh 1975) and that many cells are ouabain insensitive may be used as an argument against comparing the activity of the enzyme in the presence and the absence of ouabain.

MATERIALS

The source and grade of all the materials used in this section is given in Chapter I.

METHODS

Cell culture, plasma membrane isolation and inorganic phosphate release from the enzyme assays were all performed as outlined in Chapter I. The various experiments comprising this section were performed as detailed below.

Ion stimulation

Since the asymmetry of ions across the membrane is destroyed during membrane isolation, Na^+ and K^+ will compete with one another at their respective sites. The optimal concentration of each ion was therefore obtained by altering its concentration while keeping the concentration of the other constant. In the range over which these ions were varied the formation of NaATP^{3-} and KATP^{3-} would not be large enough to reduce the MgATP^{2-} concentration to levels which would become rate limiting.

Timecourse

Using the optimal concentrations of Na^+ and K^+ obtained

in the above experiments the linearity of the reaction was tested. Reaction mixtures were incubated at 35°C for various times and the product formation was estimated in the presence and the absence of Na^{+} and K^{+} . These results were not significantly different from those obtained when the activity was estimated with Na^{+} and K^{+} in the presence and absence of optimal concentrations of ouabain.

Inhibitors

Ouabain:- The reaction mixture was incubated in the presence and absence of ouabain for 5 min. at room temperature prior to the assay of enzyme activity during a 30 min. incubation at 35°C . The activities observed in the presence of various concentrations of ouabain were compared to the rates of the reaction when membrane was incubated in the absence of ouabain and in the presence and the absence of Na^{+} and K^{+} . The results were expressed as Dixon Plots where the abscissa is ouabain concentration and the ordinate is the reciprocal of the rate of reaction. The slope of this plot is equal to $1/V_{\text{max}} \cdot K_i$. V_{max} was obtained as detailed below and used to calculate K_i .

Adenine nucleotides:- These substances were added to the reaction mixture at the start of the reaction. After 30 min. at 35°C the reaction was terminated and the reduction in activity induced by the adenine nucleotide was expressed in terms of a percentage decrease from control values.

Kinetics

The MgATP^{2-} concentration in these experiments was

calculated by quadratic equation using the data for the dissociation constants given by Phillips (1966). The Mg^{++} and ATP^{4-} concentrations were altered so that the free Mg^{++} concentration was held constant at 0.2mM. The rate of reaction at each substrate concentration was measured over a 30 min. period and the data obtained were graphed as S/V against S . This linear transformation of the Michaelis Menten equation has a slope of $1/V_{max}$ and an intercepts of K_m/V_{max} .

Divalent cations

The concentration of divalent cation and ATP required to yield a final ATP-divalent cation complex concentration of 0.8mM was calculated according to the dissociation constants given by Philips (1966). The rate of reaction was monitored over a 30min. timecourse at 35°C and the activity obtained in the presence and absence of 1.0mM ouabain was compared to the activity observed with $MgATP^{2-}$.

RESULTS

Stimulation by sodium and potassium ions

The effect of variations in the concentrations of Na^+ and K^+ on the activity of the enzyme from 3T3 and SV40 3T3 cells is shown in Figs. 2.1-2.4. When the concentration of Na^+ was maintained at 60mM the (Na+K)-ATPase from both 3T3 (Fig. 2.1) and SV40 3T3 cells (Fig. 2.2) exhibited maximal activity in the presence of 8-10mM K^+ . At concentrations of K^+ greater than 10mM the activity became markedly reduced. In the presence of 10mM K^+ , maximal activity was obtained with 60mM Na^+ in both 3T3 (Fig. 2.3) and SV40 3T3 (Fig. 2.4) cell plasma membranes. At concentrations of Na^+ greater than 60mM the activity was reduced. The optimal concentrations of Na^+ and K^+ in both cases were 60 and 10mM respectively. These concentrations were used throughout the following experiments.

Linearity

The (Na+K)-ATPase activity from both 3T3 and SV40 3T3 cells was linear for at least 30 min. (Figs. 2.5 and 2.6). All the following experiments were performed with a concentration of membranes designed to produce measurements on the linear portion of these curves.

Inhibitors

The inhibitory effects of ouabain are shown in Figs.

Fig. 2.1 K^+ stimulation of the (Na+K)-ATPase
from 3T3 cells.

K^+ was varied from 2 to 18mM while the Na^+ concentration
was fixed at 60mM.

The specific activity of the enzyme was observed to
increase with K^+ concentration from 0 to 6mM and
then decrease as the concentration exceeded 12mM.

The concentration necessary for half maximal
stimulation was approximately 0.5mM K^+ .

The points are the mean of 3 experiments performed
in duplicate and the vertical bars represent the
standard error of the mean.

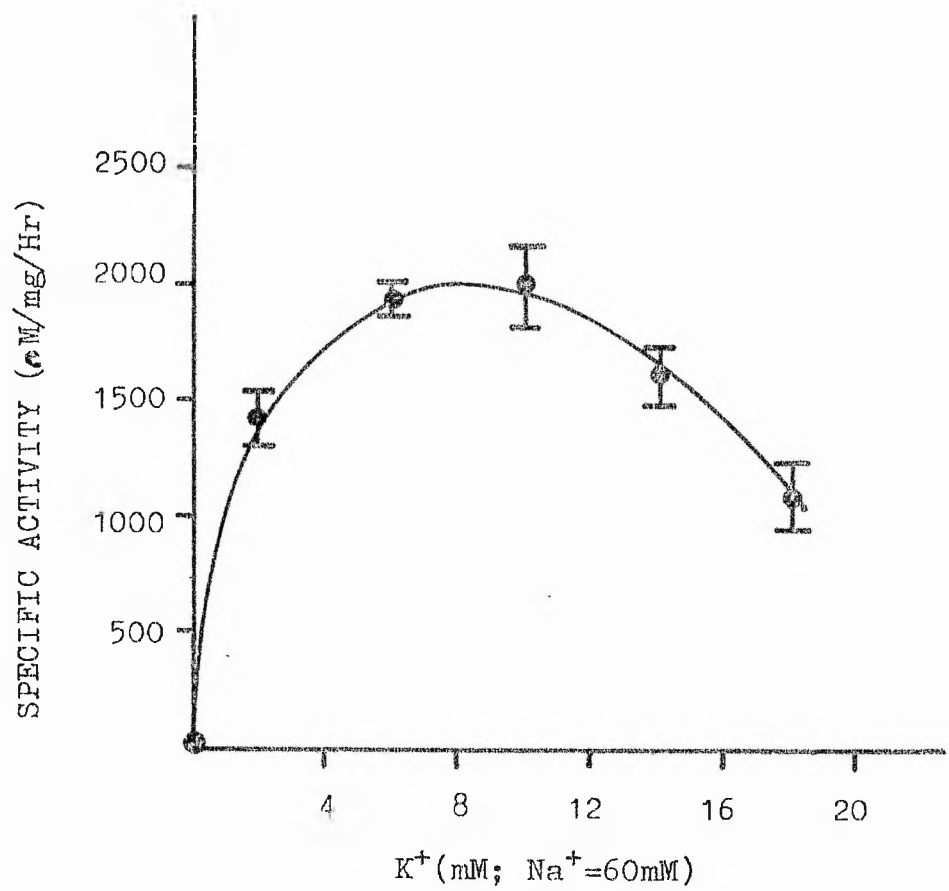


Fig. 2.1 Potassium stimulation of the (Na+K)-ATPase from 3T3 cells.

Fig. 2.2 K^+ stimulation of the (Na+K)-ATPase from SV40 3T3 cells.

K^+ was varied from 0 to 18mM while the Na^+ concentration was fixed at 60mM.

The specific activity of the enzyme was observed to increase with K^+ concentration unto 10mM and to decrease as the $[K^+]$ exceeded this value.

The concentration of K^+ necessary for half maximal stimulation is approximately 0.2mM.

The points are the mean of 3 experiments performed in duplicate and the vertical bars represent the standard error of the mean.

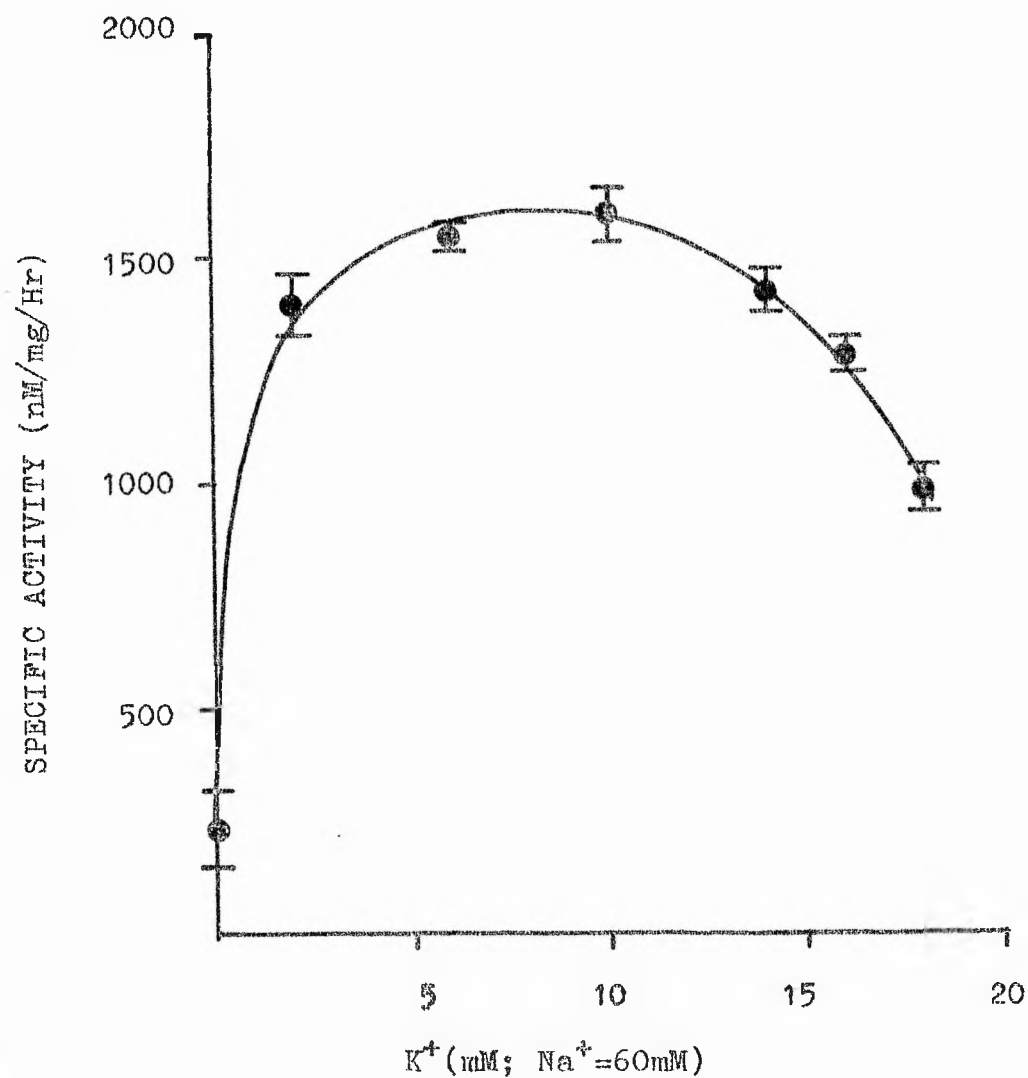


Fig. 2.2 Potassium stimulation of the
(Na+K)-ATPase from SV40 3T3 cells

Fig. 2.3 Na^+ stimulation of the (Na+K)-ATPase from 3T3 cells.

The K^+ concentration was fixed at 10mM and the Na^+ concentration was increased from 0-120mM. The specific activity of the enzyme increased up to 60mM and, above this value, declined gradually. The Na^+ concentration necessary for half maximal stimulation of the enzyme is approximately 16mM.

The points represent the mean of three experiments performed in duplicate and the vertical bars represent the standard error of the mean.

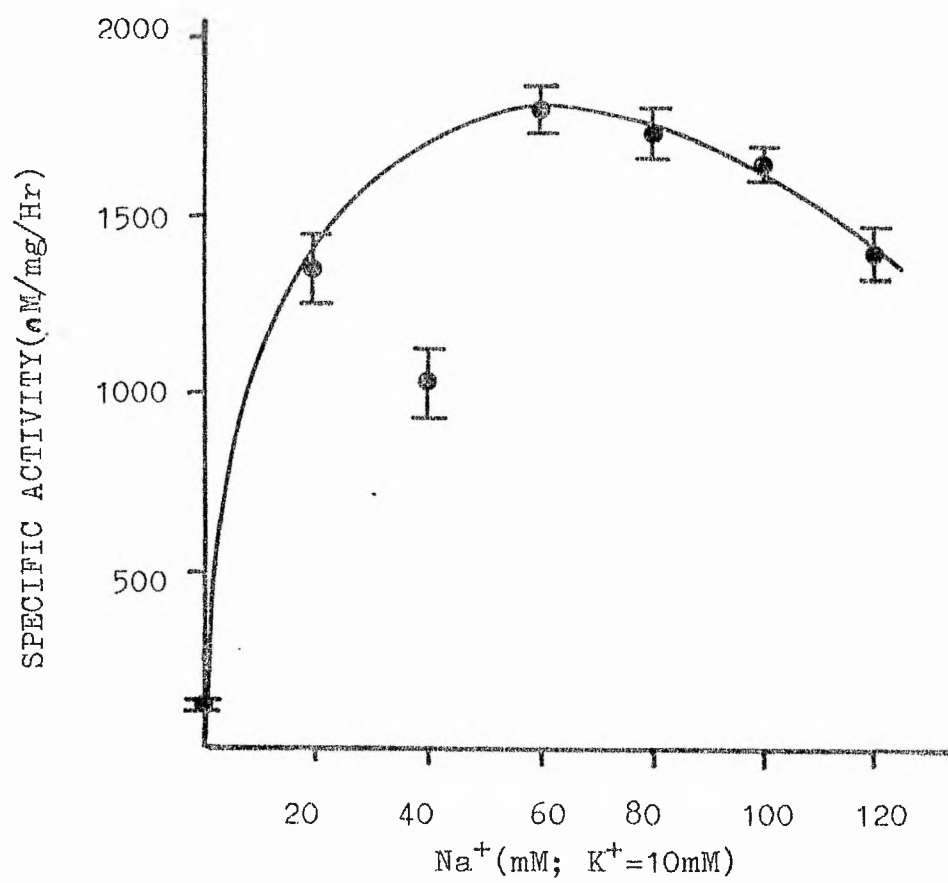


Fig.2.3 Stimulation by Na⁺ of the (Na+K)-ATPase from 3T3 cells.

Fig. 2.4 Na^+ stimulation of the (Na+K)-ATPase from SV3T3 cells.

The Na^+ was varied from 20-120mM while the K^+ concentration was fixed at 10mM. As the Na^+ concentration increased the specific activity increased up to a maximum at 60mM and was reduced as the concentration was further increased.

The concentration necessary for half maximal stimulation is approximately 21mM.

The points represent the mean of three experiments performed in duplicate and the vertical bars represent the standard error of the mean.

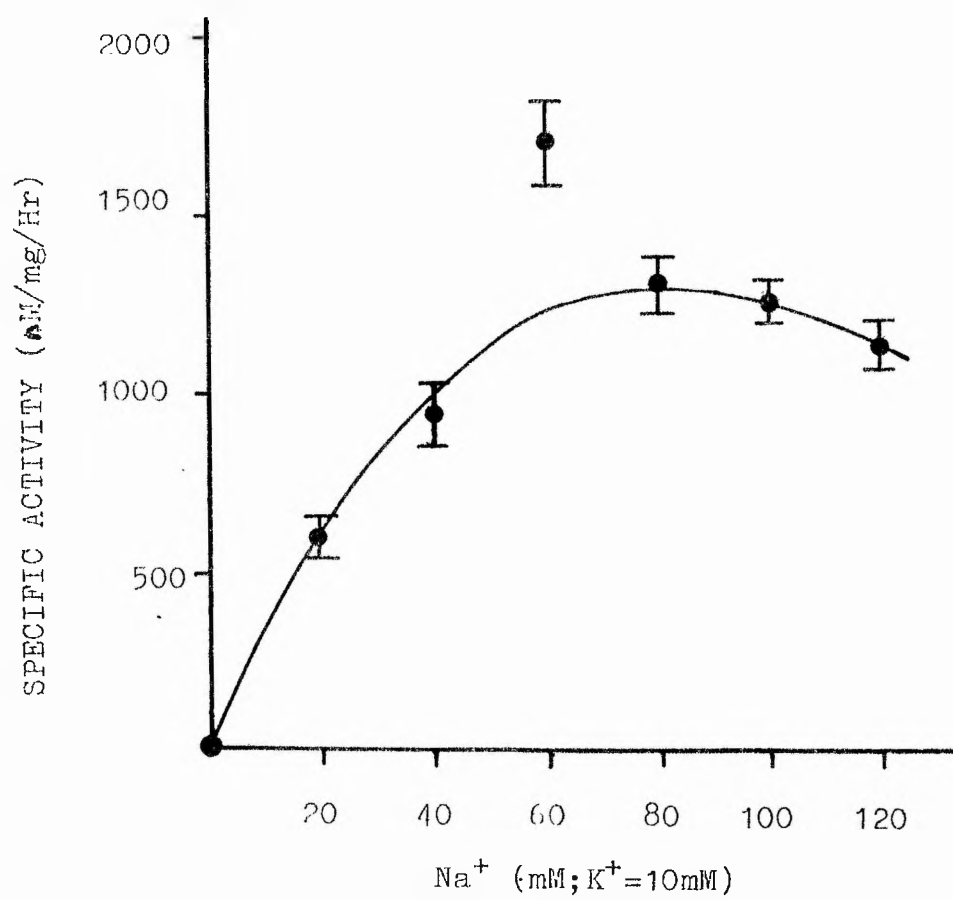


Fig.2.4 Sodium stimulation of the (Na+K)-ATPase from SV₄₀3T3 cells.

Fig. 2.5 Timecourse of hydrolysis of ATP by the (Na+K)-ATPase of 3T3 cells.

The rate of the Na^+ and K^+ stimulated hydrolysis of 0.8mM MgATP^{2-} is linear for atleast 30 mins. The activity was measured in the presence and absence of 10 mM KCl and 60 mM NaCl.

The values given are the mean of three experiments performed in duplicate \pm the standard error of the mean.

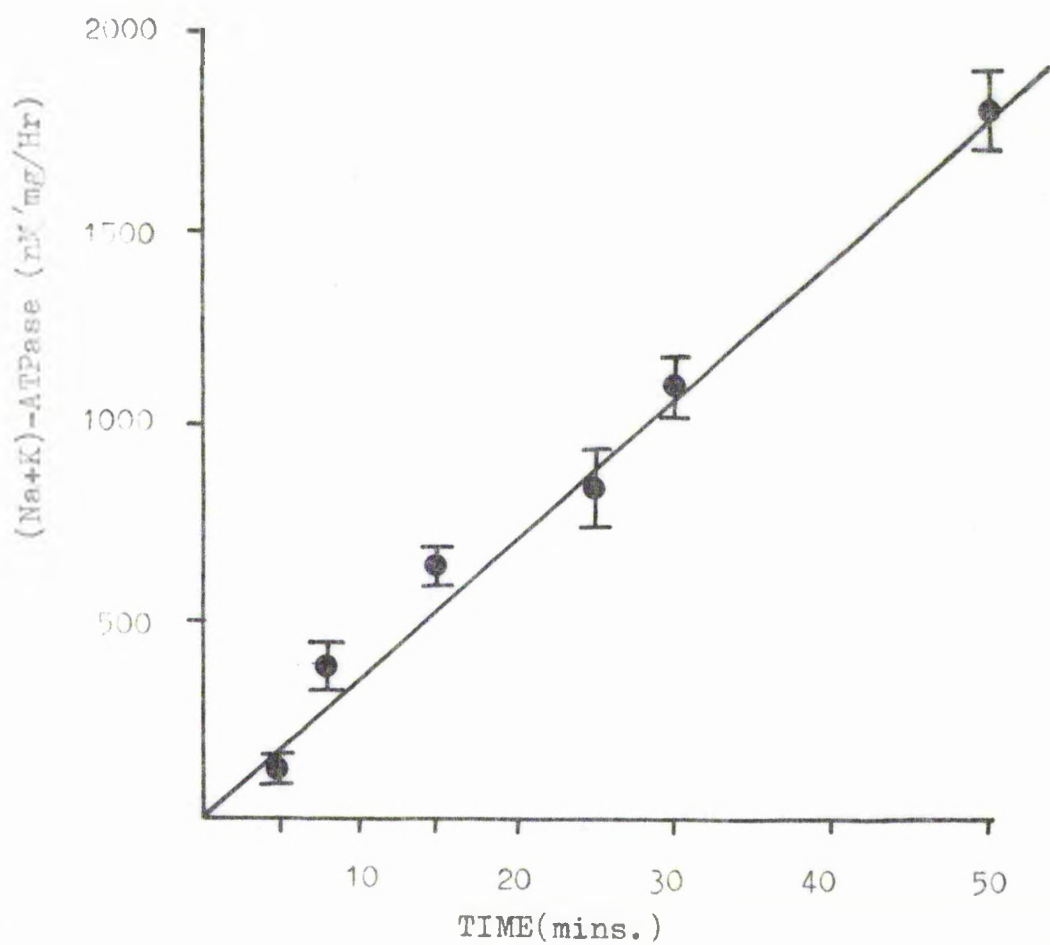


Fig. 2.5 Timecourse of the (Na+K)-ATPase from 3T3 cells.

2.7 and 2.8. The (Na+K)-ATPase from 3T3 cells (Fig. 2.7) appears to be more sensitive to inhibition by ouabain than that from SV40 3T3 cells (Fig. 2.8). In SV40 3T3 cells the K_i for ouabain is twice that of 3T3 cells and the concentration of ouabain necessary for maximal inhibition is 1.0mM compared to 0.5mM.

The effect of various adenine nucleotides on the rate of reaction was also examined (Table 2.1). Adenyl cyclase in the plasma membrane will produce 3'5' cyclic AMP (cAMP) from ATP, while the ATPase will produce ADP which may be further hydrolysed to AMP and adenosine. Any or all of these end products may be responsible for the loss of linearity which is observed beyond a 30 min. incubation period. The effect of these substances is shown in Table 2.1. AMP and adenosine have no significant effect on the (Na+K)-ATPase in 3T3 cells and AMP does not affect the activity in SV40 3T3 cells but adenosine reduces the latter by some 30%. cAMP and ADP at concentrations of 0.5mM and 1.0mM have similar effects on the activity of the enzyme in 3T3 cells, while the SV40 3T3 cells are less sensitive to inhibition by these compounds.

Enzyme kinetics

Figs. 2.9 and 2.10 show plots of S/V against S for SV40 3T3 and 3T3 cells. The V_{max} of the enzyme from 3T3 cells is significantly greater than that of SV40 3T3 cells and the K_m for the latter is two fold greater than the former.

Fig. 2.6 Timecourse of hydrolysis of ATP by the (Na+K)-ATPase of SV403T3 cells.

The rate of hydrolysis, as was the case with 3T3 cells, in the presence of 10.0mM KCl and 60mM NaCl was linear over at least the first 30 mins of incubation.

The values given are the mean of three duplicate observations \pm the standard error of the mean.

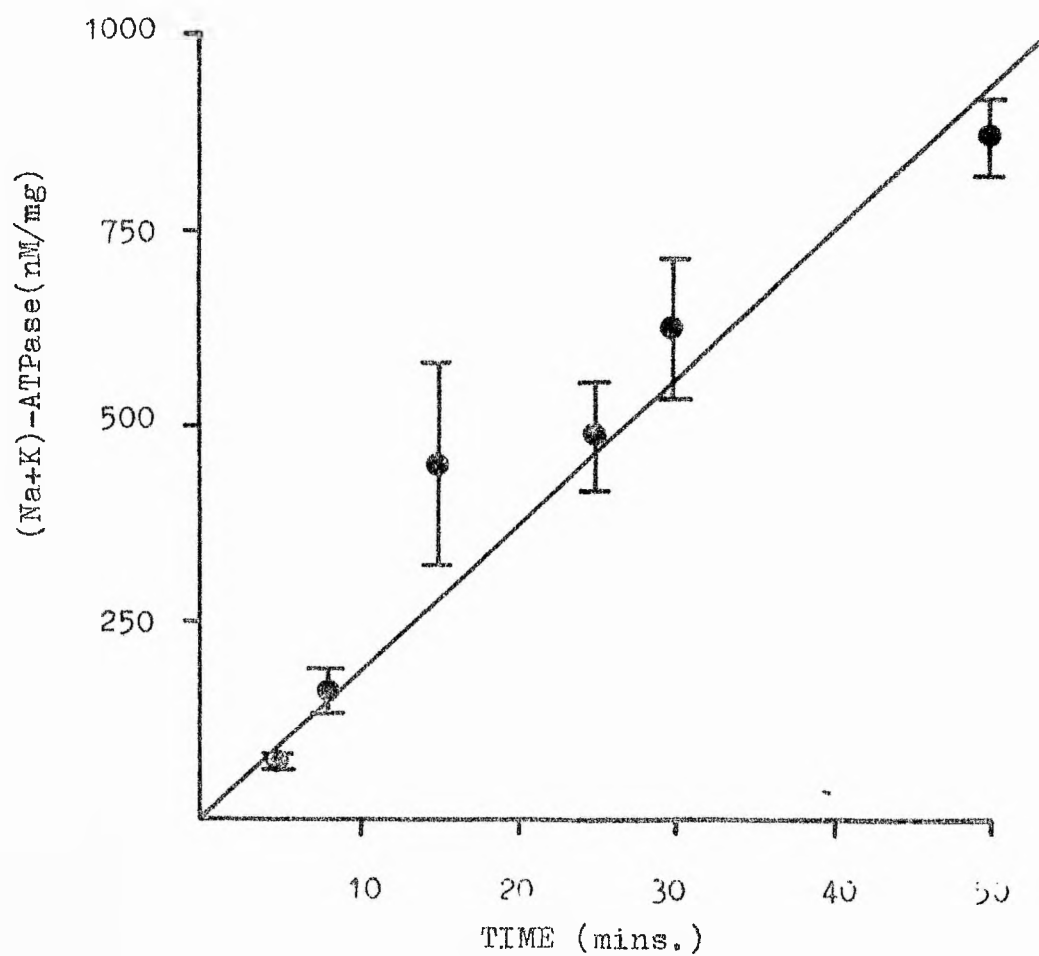


Fig. 2.6 Timecourse of the (Na+K)-ATPase from SV40 3T3 cells.

Fig. 2.7 Ouabain inhibition of the (Na+K)-ATPase from 3T3 cells.

The slope of the Dixon plot shown on the opposite page is equal to $1/V_{max} \cdot K_i$.^{*} Using the value for V_{max} ($1960 \pm 30 \text{ nM/mg/hr}$) obtained from fig. 10, this yields a K_i of ouabain for the enzyme of $0.04 \pm 0.005 \text{ mM}$.

The points are the mean of three experiments performed in duplicate and the vertical bars represent the standard error of the mean. The slope of the line was calculated by linear regression analysis and the line was drawn by eye.

* MAHLER, H.R. & CORDES, E.H. (1968)

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HARPER & ROW, NEW YORK.

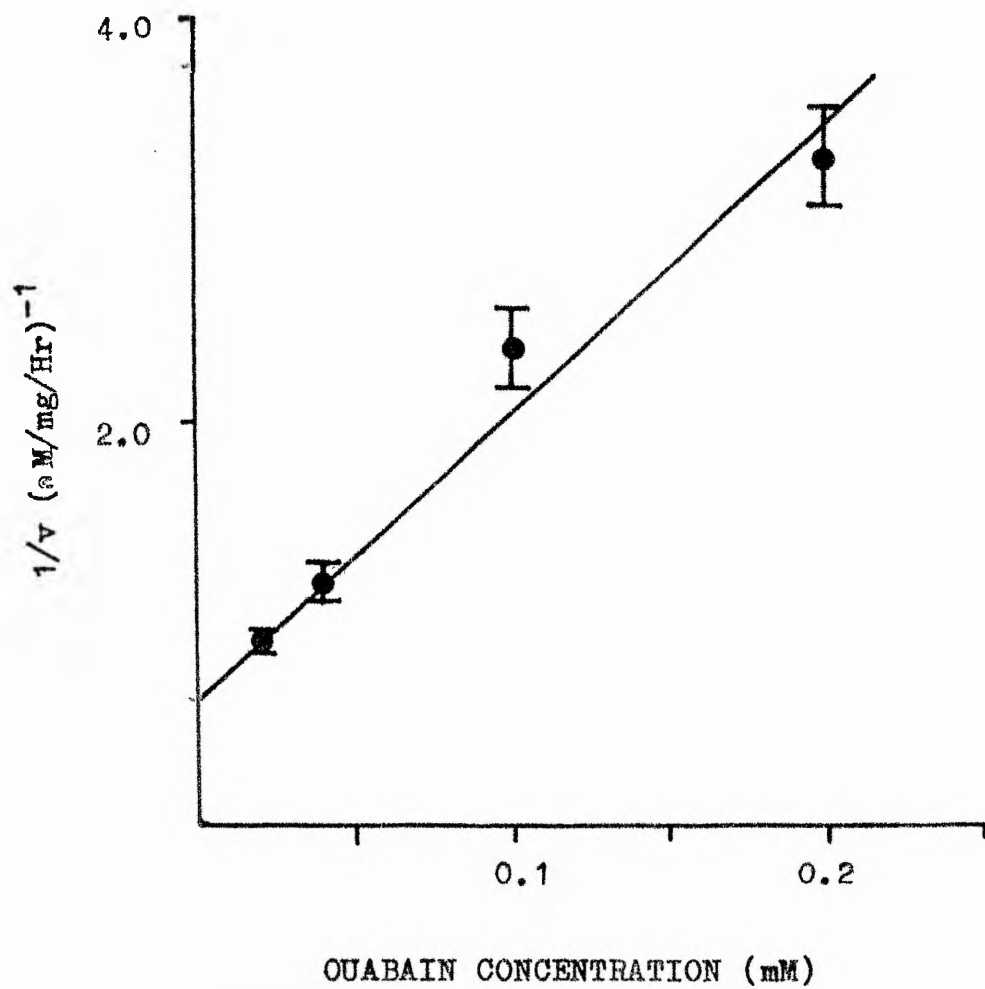


Fig. 2.7 Ouabain inhibition of the (Na+K)-ATPase from 3T3 cells.

Fig. 2.7 5-6-86.

Fig. 2.8 Ouabain inhibition of the (Na+K)-ATP
ase from SV40 3T3 cells.

The slope of the Dixon plot* shown on the opposite page is equal to $1/V_{max} \cdot K_i$. * Given the value obtained for V_{max} in fig. 2.9, the K_i of ouabain for the enzyme is $0.085 \pm 0.008 \text{ mM}$. This value is significantly greater than that obtained for 3T3 cells ($p=0.01$ from the students T test).

The points are the mean of 3 experiments performed in duplicate and the vertical bars represent the standard error of the mean. The slope of the line was calculated by linear regression analysis and the line was drawn by eye.

* SEE 2.7.

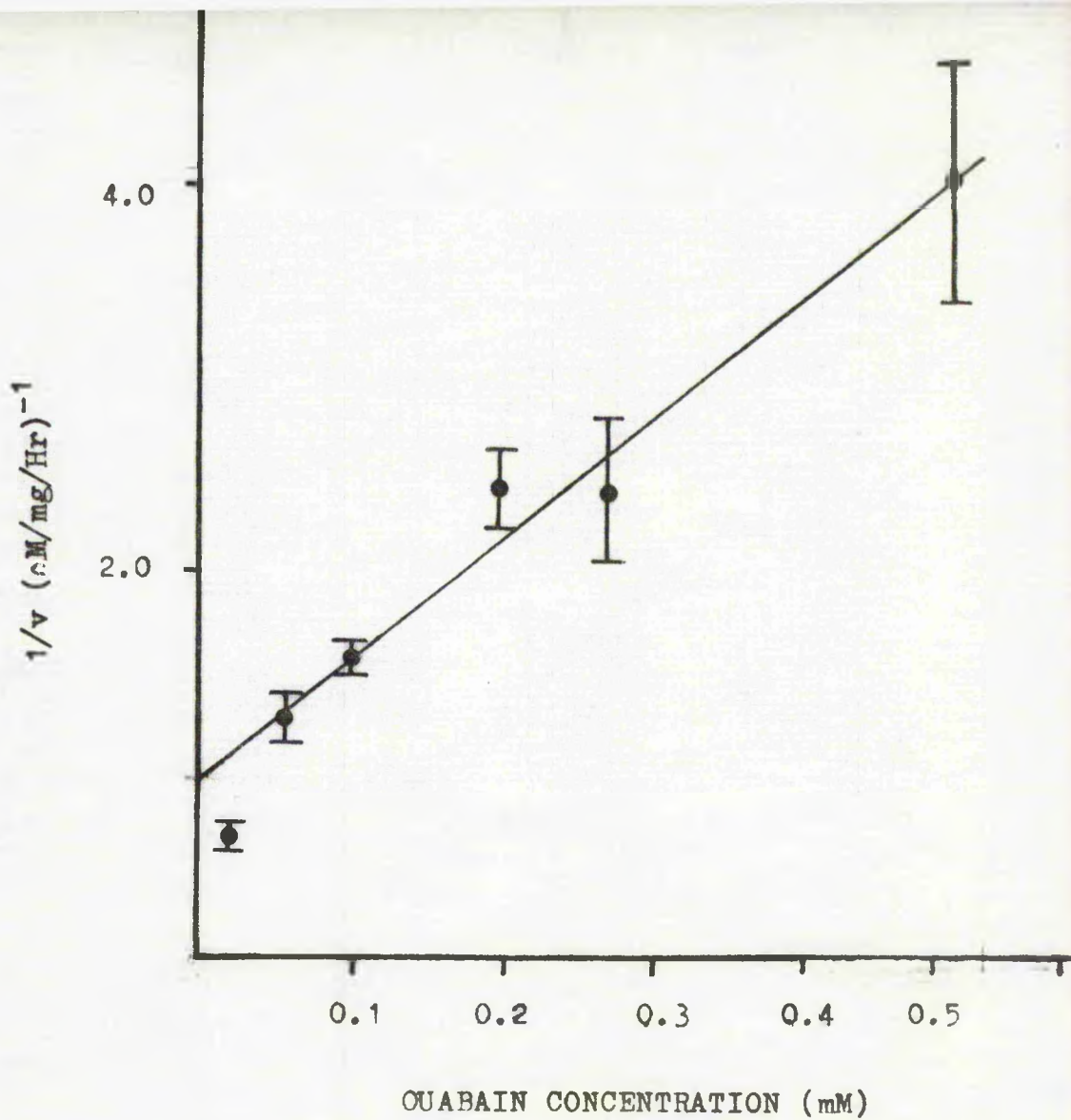


Fig. 2.8 Ouabain inhibition of the (Na+K)-ATPase from SV40 3T3 cells.

eyeballed 0.09

Fig. 2.9 Woolf plot of the kinetics of the (Na+K)-ATPase from SV40 3T3 cells.

In this linear transformation of the Michaelis-Menten equation, the slope of the line = $1/V_{\max}$ and the intercept = K_m/V_{\max} . In this case $K_m = 0.07 \pm 0.003 \text{ mM}$ and $V_{\max} = 1600 \pm 100 \text{ nM/mg/Hr}$. The activity was measured over a 30 min. period, the line was drawn by eye and the slope and intercepts were calculated by linear regression analysis. The MgATP^{2-} concentration was calculated as described in the methods section.

The points are the mean of 3 experiments and the vertical bars are the standard errors of the means.

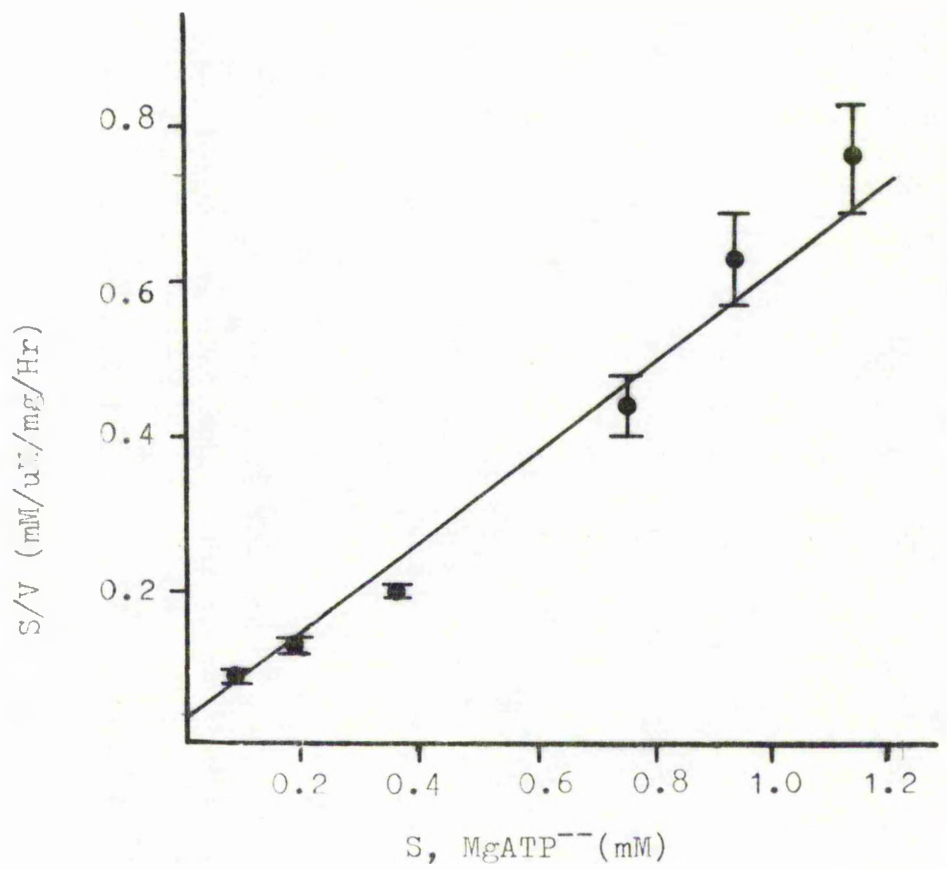


Fig.2.9 Kinetics of the (Na+K)-ATPase from SV₄₀ T3 cells.

K_m by eye 0.066

V_{max} 1.680

v
 $1/v$

more accurate $\rightarrow K_m$ 0.08 ± 0.02
 V_{max} 1.450 ± 0.500

Fig. 2.10 Woolf-Eadie plot of the kinetics of the (Na+K)-ATPase from 3T3 cells.

The activity was measured over a 30 min period. The line was drawn by eye and the slope and the intercepts were calculated from linear regression analysis. The MgATP^{2-} concentration was calculated as described in the methods section.

In this case $V_{\text{max}} = 1960 \pm 30 \text{ nM/mg/Hr}$ and the $K_m = 0.13 \pm 0.01 \text{ mM}$.

The points are the mean of three experiments performed in duplicate and the vertical bars represent the standard error of the mean.

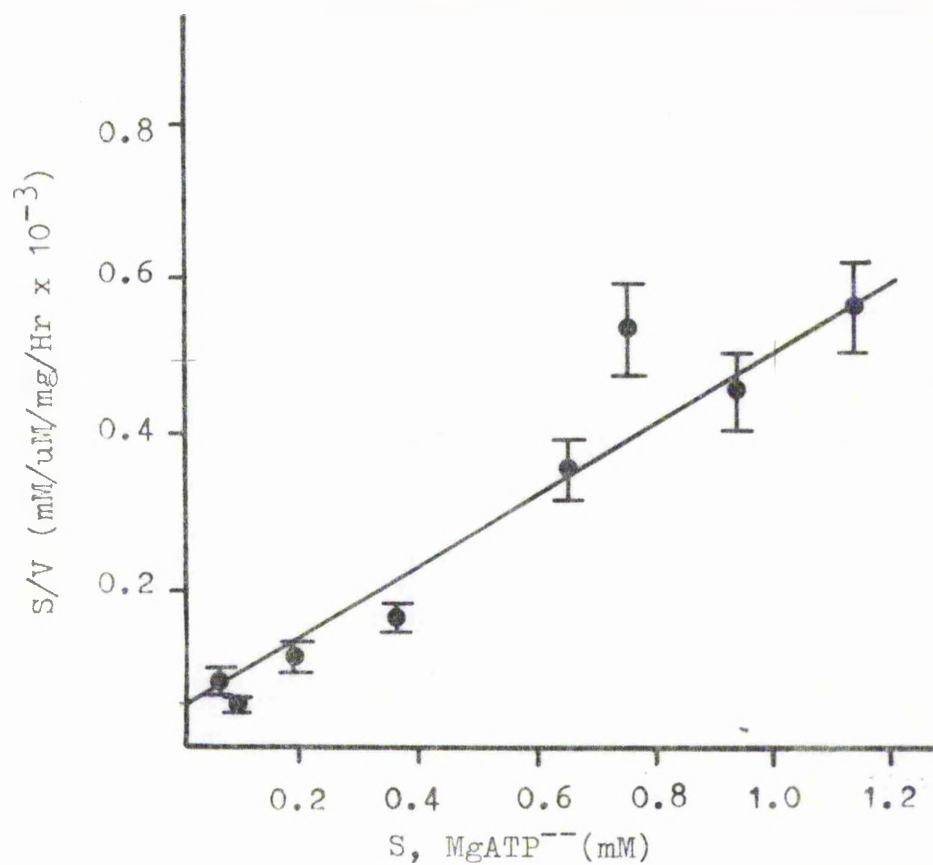


Fig. 2.10 Kinetics of the (Na+K)-ATPase from 3T3 cells.

$V_{max} = 2034$
 $K_m = 0.13$

$V_{max} = 2400 \pm 400$
 $K_m = 0.09 \pm 0.04$

Hofstee ✓

$\frac{V}{S}$

Table 2.1 The effect of various adenine nucleotides on the activity of the (Na+K)-ATPase from 3T3 and SV3T3 cells.

AMP at concentrations up to 1mM had no significant effect on either enzyme. Adenosine had no effect on the 3T3 (Na+K)-ATPase. ADP depressed the activity of both enzymes to the same extent. 3T3 cells were more susceptible to inhibition by cAMP and SV3T3 cells were more susceptible to inhibition by adenosine.

The 100% activity values represent 1960 nM/mg/Hr for 3T3 cells and 1600 for SV3T3 cells.

Hydrolysis was estimated over a 30 min period in the presence of 60mM NaCl and 10mM KCl in the presence and absence of 1mM ouabain.

Values are the mean of three experiments performed in duplicate \pm the standard error of the mean.

TABLE 2.1 Adenine nucleotides as inhibitors of the
(Na+K)-ATPase from SV40 3T3 and 3T3 cells.

<u>Inhibitor</u>	<u>Concentration(mM)</u>	Activity as a percentage of control	
		<u>SV40 3T3</u>	<u>3T3</u>
AMP	0.5	100 \pm 5	100 \pm 8
	1.0	100 \pm 7	100 \pm 10
3'5'cyclic AMP	0.5	92 \pm 4	73 \pm 8
	1.0	81 \pm 3	55 \pm 7
ADP	0.5	76 \pm 8	77 \pm 4
	1.0	67 \pm 10	56 \pm 8
Adenosine	0.5	73 \pm 5	100 \pm 4
	1.0	78 \pm 5	100 \pm 6

Divalent cations

Of the various divalent cation ATP complexes used none was hydrolysed as rapidly as MgATP^{2-} (Table 2.2). For both 3T3 and SV40 3T3 cell (Na+K)-ATPases the effectiveness with which these ions could replace Mg^{2+} decreased in the following way $\text{Mg}^{2+} > \text{Mn}^{2+} > \text{Ca}^{2+} > \text{Be}^{2+} \simeq \text{Ba}^{2+} \simeq \text{Zn}^{2+}$.

pH Optimum

The effect of alterations in pH on the activity of the (Na+K)-ATPase from SV40 3T3 cells is shown in Fig. 2.11. The pH optimum for the enzyme was found to be 7.6. On either side of this value the activity was reduced. The range was not extended beyond the limits in the diagram since, beyond these values the MgATP^{2-} concentration is greatly reduced by the formation of other ionic species.

The data obtained from these experiments were used in the estimation of enzymic activity as the number of cells/surface area increased.

Table 2.2 The hydrolysis of various divalent cation ATP complexes by the (Na+K)-ATPase of 3T3 and SV3T3 plasma membranes.

The activity of the enzyme was highest with Mg^{++} and decreased in order $Mg^{++} > Mn^{++} > Ca^{++} > Be^{++} \simeq Ba^{++} \simeq Be^{++}$. The trend was the same in both cases.

For 100% activity values see Fig 2.1.

Values are the mean of three experiments performed in duplicate \pm the standard error of the mean.

TABLE 2.2 Divalent cation ATP complexes as substrates
for the (Na+K)-ATPase from SV40 3T3 and 3T3 cells.

<u>Substrate</u>	Activity as a percentage of control	
	<u>SV40 3T3</u>	<u>3T3</u>
MgATP ²⁻	100	100
MnATP ²⁻	62 ± 10	51 ± 10
CaATP ²⁻	22 ± 8	34 ± 6
BeATP ²⁻	3 ± 2	zero
BaATP ²⁻	zero	zero
NiATP ²⁻	zero	zero

Fig. 2.11 The effect of pH on the activity of the (Na+K)-ATPase from SV40 3T3 cells.

Maximal activity was obtained at pH 7.6 and, as the pH was increased above or decreased below this value, the activity was reduced.

The activity was estimated over a 30 min. period, in the presence of 60mM NaCl and 10mM KCl and in the presence or absence of 1.0mM ouabain. The activity is expressed in terms of a percentage of the maximum activity observed.

The values given are the mean of 3 experiments performed in duplicate and the vertical bars represent the standard error of the mean.

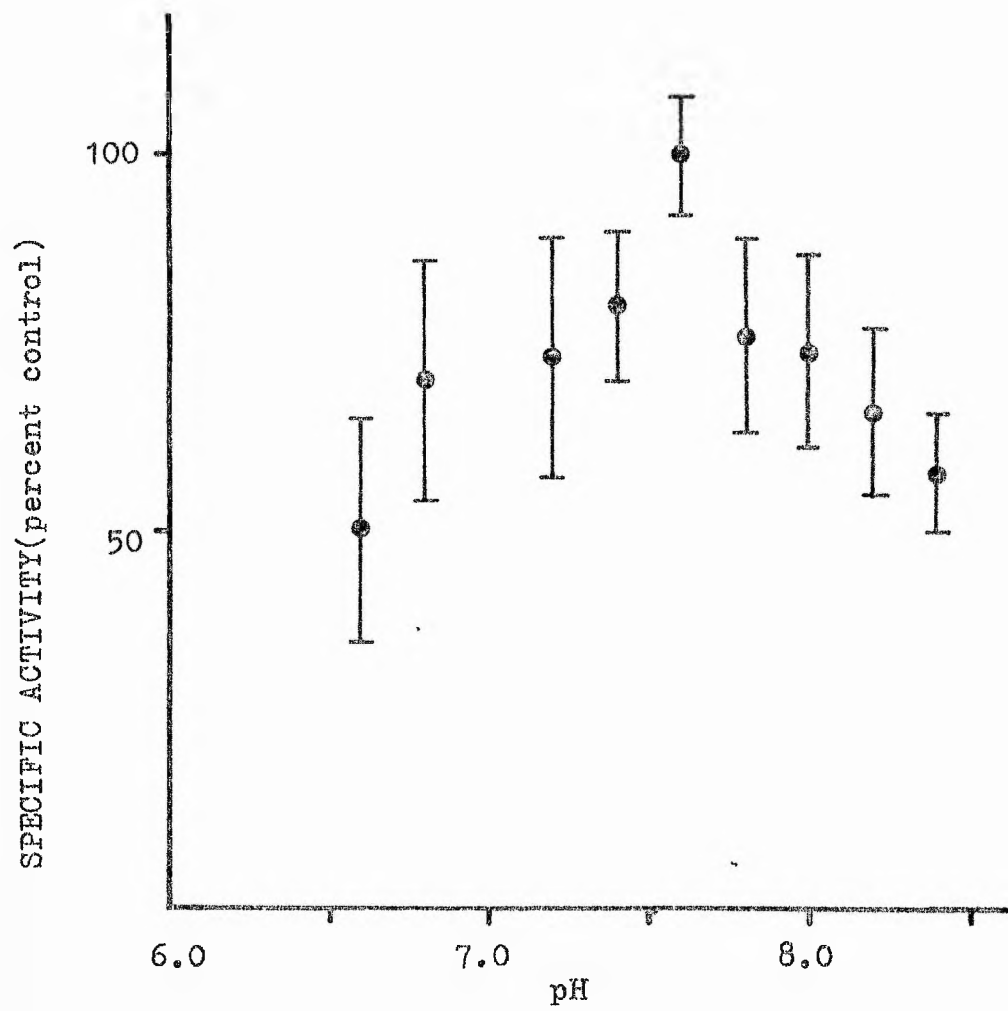


Fig. 2.11 The effect of pH on the rate of hydrolysis of ATP by the (Na+K)-ATPase of SV40 3T3 cells.

DISCUSSION

The properties of the (Na+K)-ATPase from various sources seem to be very similar in terms of structure and ionic, substrate and inhibitor profiles. The (Na+K)-ATPases of 3T3 and SV40/3T3 cells are no exception. The concentration for half maximal activation of the enzyme by Na⁺ and K⁺ and the concentration of ouabain necessary for half maximal inhibition are within the normal range (Glynn and Karlish 1975).

The activity of both of the enzymes is linear over at least 30 min. and the specific activity of the enzymes from 3T3 cells is greater than that of the SV40 3T3.

Ouabain inhibits the activity of both enzymes but the 3T3 (Na+K)-ATPase appears slightly more sensitive. The activity of the latter is completely inhibited by 0.5mM ouabain while 1.0mM is necessary to inhibit the enzyme in SV40 3T3 cell plasma membrane completely. Ouabain at these concentrations did not significantly effect the activity of the MgATPase in the absence of Na⁺ and K⁺. No stimulation of activity was observed at low concentrations of ouabain (Bonting 1970), but perhaps the concentrations used in the present investigations were not low enough to elicit this response.

In the case of both enzymes the activity increases initially in Na⁺ and K⁺. At levels greater than those required to produce maximal stimulation, the activity is

reduced. As previously mentioned, in an isolated plasma membrane the normal ionic asymmetry will be lost, so that Na^+ in addition to stimulating the enzyme at the internal binding site, will competitively inhibit K^+ at the external binding site. Similarly K^+ will inhibit Na^+ binding to the internal site as well as having a stimulatory effect on the external binding site.

The levels of Na^+ and K^+ used in these experiments were unlikely to reduce the MgATP^{2-} concentration to an extent which would seriously affect the rate of hydrolysis. Even in the presence of 200mM $\text{Na}^+ + \text{K}^+$ the final MgATP^{2-} concentration would be 0.68mM. Since the K_m (apparent) for the enzymes are 0.13 and 0.07mM MgATP^{2-} , the initial rates of hydrolysis at 0.68mM should only be very slightly lower than that at 0.8mM.

The observation that hydrolysis of the CaATP^{2-} by the plasma membrane fraction was stimulated by Na^+ and K^+ and inhibited by ouabain was curious in view of the fact that Ca^{++} ions are thought to potently inhibit the (Na+K)-ATPase (Tobin et al 1973). The stimulatory effect of chelating agents has been briefly reviewed by Duck-Chong (1976) who suggests that the activation of the (Na+K)-ATPase in untreated membranes by phosphatidyl serine might be due to the chelation of Ca^{++} by this compound. Experiments reported in Chapter I of this thesis suggest that the (Na+K)-ATPase activity of these cultured cells could not be observed in the absence of 0.1mM EDTA. However, the activity is no less in buffer containing 0.5mM $\text{CaCl}_2 + 0.5\text{mM MgCl}_2$ than in buffer containing 1.0mM MgCl_2 . The free Ca^{++} in the former solution might be expected to

inhibit the enzyme but this is not the case. In these cells EDTA appears to exert its effect by chelating heavy metal ions other than Ca^{++} ; rather than inhibiting the enzyme CaATP^{2-} actually acts as a substrate. *-0000 changes it is confirmed*

One of the most interesting results of these experiments was the observation that the activity of the (Na+K)-ATPase from 3T3 cells was greater than that from SV40 3T3 cells. Elligsen (1974) showed that the specific activities of (Na+K)-ATPase in 3T3 and SV40 3T3 cells at low population densities were similar. At high cell densities, however, the activity of 3T3 cells fell, while the activity of SV40 3T3 cells was unaltered. At high cell densities therefore, the specific activity of the (Na+K)-ATPase in SV40 3T3 cells was less than that of 3T3 cells. However, Graham (1972) showed that after transformation by polyoma virus, the (Na+K)-ATPase activity of BHK cells was reduced. The differences may be due to serum effects. The results of the investigations described in Section IV, using different sera, have been confirmed by Mohammed (personal communication) who has also shown that, in calf serum, 3T3 cells at confluent densities have a greater specific activity of (Na+K)-ATPase than SV40 3T3 cells, while in foetal calf serum the (Na+K)-ATPase activity is greater in the latter than in the former.

The (Na+K)-ATPase of 3T3 cells is more sensitive to inhibition by cAMP than the SV 3T3 cell enzyme. It has been reported that, on confluence, the intracellular cAMP levels of 3T3 cells rise, while the levels of this compound in SV 3T3 cells are unaffected by cell density (Otten et al 1971).

If the sodium pump in situ is affected in the same manner as the enzyme, then the transport capacity of the cell for Na^+ and K^+ could be reduced at confluence, without affecting the maximal activity of the enzyme. In this way, the rate of pumping might be reduced while the enzymic activity remains unaltered or even increases.

CHAPTER III

THE EFFECT OF CONDITIONED MEDIUM ON THE
UPTAKE OF RUBIDIUM IONS INTO
3T3 AND SV40 3T3 CELLS

SUMMARY

1. The effect of pretreatment with fresh and conditioned growth medium on the total uptake of ^{86}Rb into 3T3 and SV40 3T3 cells was examined.
2. Pretreatment with conditioned medium increases the rate of ^{86}Rb uptake from Krebs into both 3T3 and SV40 3T3 cells.
3. The effect was not abolished in the absence of serum. In fact, with serum-free conditioned medium the effect was more marked ^{THAN} with serum-containing conditioned medium.
4. The conditioning effect was not abolished by boiling the conditioned medium prior to preincubation.
5. The effect of conditioned medium was abolished on dialysis.
6. When the uptake was measured directly from fresh and conditioned growth medium, it appeared greater in the former than in the latter. This observation held for both 3T3 and SV40 3T3 cells.
7. It appeared that after pretreatment with fresh growth medium, the uptakes of ^{86}Rb from Krebs and fresh

growth medium were not significantly different. However, after pretreatment with conditioned medium, the uptake from Krebs was significantly greater than from conditioned medium. It was concluded that the transfer of cells from conditioned medium to Krebs resulted in an increase in the rate of uptake. The effect was not investigated further.

8. It was observed, during the above experiments, that medium conditioned by 3T3 cells was less able than fresh medium to support the growth of other 3T3 cells. However, the growth of 3T3 cells was stimulated by medium conditioned by SV40 3T3 cells.
9. Serum free conditioned medium collected from cultures of SV40 3T3 cells acted synergistically with low concentrations of serum to promote the growth of normal cells.
10. This material could be concentrated without loss of its stimulatory effect.
11. Polyacrylamide gel electrophoresis showed that serum-free medium conditioned by 3T3 and SV40 3T3 cells had a relatively simple protein composition.

INTRODUCTION

It is generally accepted that there is a mutual interaction between cells in culture and the culture medium; cells remove substances from the medium and secrete materials into solution.

One of the first observations of the ability of cells to condition their medium was made by Earle (1948) who demonstrated that single mouse cells even in small quantities of medium (horse serum supplemented with chick embryo extract) would only grow after a few days' delay. It was concluded that the medium was initially inadequate to support cell growth and must first be modified by detoxification, alteration of the redox potential or secretion of growth promoting substances or extracellular enzymes. Pace and Aftonomos (1957) showed that the minimum inoculum concentration—the minimum number of cells which must be plated for growth to occur - of mouse liver cells was reduced from 90 000 cells/ml. to 10 000 cells/ml. when conditioned medium was used to support growth.

Chick embryo fibroblasts may also condition their own growth medium, in this case, by laying down a carpet of 'microexudate' which enhances the growth of sparsely seeded cells (Igarashi and Yaoi 1975). A growth promoting substance could also be released from these cells by treatment with 1M urea in 5mM EDTA.

A factor which promotes the growth of fibroblasts (FGF) may be obtained from bovine brain and pituitary gland.

This factor, together with hydrocortisone, will completely replace serum in the induction of cell division in BALB/c 3T3 cells, but does not stimulate cells transformed by simian virus (Rutland et al 1974). Rat liver appears to be a source of transformed fibroblast growth factor (TFGF, Lipton et al 1975) which stimulates the division of SV40 3T3 cells but not 3T3 cells. Both FGF and TFGF are heat labile and rendered inactive by treatment with pronase but not RNAase or DNAase. TFGF is reported to have similar heat and pH stability to the SV40 3T3 growth promoting factor found in serum. That TFGF and FGF are not the same molecular species is corroborated by the observations of Holley and Kiernan (1971), that the serum factor required by SV40 3T3 cells is not that which is rate limiting for the growth of 3T3 cells.

Trypsinised cells bind large quantities of serum proteins (Knox and Pasternak 1977) and during subsequent trypsinisation more than 60% of this protein may be released from these cells. It is interesting to speculate that the binding of these proteins to the cell surface might induce the rapid alterations in transport of certain substances which are observed when cultures are treated with fresh medium (see Chapter IV). Knox and Pasternak (1977) provide evidence to suggest that cells bind to their surfaces substances which stimulate their growth. When cells are treated with serum free medium, after they have completed their round of division, they divide slowly if at all. However, when cells, in serum free medium, which are not dividing are treated with serum, immediately washed and reincubated in serum free medium, they are

stimulated to divide. In addition, when cells are centrifuged through serum they deplete its ability to stimulate growth.

Although transformed cells possess the ability to grow in conditions of low serum, this ability is not due to an increased protein binding. These cells, on a cell to cell basis, bind no more protein than normal cells. The growth requirements of SV40 3T3 cells are less stringent; these cells will grow in conditions which do not permit the growth of 3T3 cells. In serum free medium to which has been added the serum protein, fetuin, both 3T3 and SV40 3T3 cells will elongate but only SV40 3T3 cells will divide. Even the addition of fraction V albumin induces only a few 3T3 cells to divide.

Since cells in culture are thought to both bind and release protein, it is difficult to determine whether proteins found in serum free cell washes have been synthesised de novo or result from the release of some previously bound serum component. There have been reports of cultured fibroblasts producing albumin and gamma globulin. When Halpern and Rubin (1970) grew cells in the presence of labelled amino acids they found that chick cells released proteins, containing ^3H or ^{14}C amino acids, which possessed the same antigenic determinants as albumin and gamma globulin from chick serum but were in fact different proteins. Results of this type are, however, equivocal, since labelled protein will arise both from de novo synthesis within the cell and from the binding of amino acids to serum proteins, a phenomenon which is well documented.

All of the effects of conditioned medium cannot be ascribed to the release of previously bound serum proteins, since the medium from cultures of some transformed cells will support the overgrowth of normal cells (Rubin 1970). Given that cells deplete the growth medium of their own specific growth factors, the growth promoting factors in conditioned serum free medium must have been released into the medium or must arise from the action of the cells on serum factors which have been bound previously.

The effect of conditioning on cellular transport mechanisms has been a much neglected field. However, Hume and Lamb (1974) showed that conditioned medium could bring about an increase in the rate of amino acid uptake into HeLa cells and Xenopus cells in culture. Subsequent observations by Brown et al (1975) using Py 3T3 cells, demonstrated that the effect of conditioned medium was to increase the rate of uptake of $^{86}\text{Rb}^+$ and ^3H 2-deoxy glucose. It was suggested that the cells released into their medium, dialysible substances which caused an increase in the activity of several of their transport systems.

At first sight these results were curious in view of the reports in the literature on the apparent stimulation of both glucose analogue and rubidium uptake by the application of fresh serum or growth medium. Lever et al (1976) have reported an increase in ouabain sensitive rubidium influx into quiescent mouse fibroblasts on the addition of serum to the growth medium. Tupper et al (1977) have reported a similar phenomenon. However, these authors showed an increase in Rubidium ion uptake only into normal

cells and the serum was reported to have no effect on the transformed varieties.

If the ability of conditioned medium to stimulate the sodium pump were limited to the transformed cells, then this might explain why these cells do not show a decreased transport capacity as the density of their cultures increase (Brown 1976).

MATERIALS

Ficoll (type 400) and coumassie blue were obtained from the Sigma Chemical Company Ltd. Acrylamide, NN'Methylene-bisacrylamide (BIS.) and TEMED (N,N,N'N'-tetramethylethylenediamine) were obtained from British Drug House Ltd. and bromophenol blue was obtained from Bio-Rad Laboratories Ltd. $^{86}\text{RbCl}$ (1mCi/ml) was obtained from the Radiochemical Centre, Amersham and the grade and source of all other reagents is given in Chapter I.

METHODS

Cell culture.

As described in Chapter I.

Rubidium uptake

$^{86}\text{Rb}^+$ will substitute for K^+ in the operation of the sodium pump (Lamb et al 1973; Boardman et al 1974; Culf and Lichtman 1975). The use of the $^{86}\text{Rb}^+$ isotope is advantageous, since its half life of 18.5 days, which is considerably longer than ^{42}K , makes it unnecessary to correct for isotope decay during counting.

Cultures to be used for measurement of rubidium uptake were grown in petri dishes. At the appropriate time the growth medium was removed and replaced with either fresh or

conditioned growth medium and incubated for 30 min. at 37°C in an atmosphere of 95% air 5% CO₂. The media was then decanted, the cultures were washed with Krebs solution at 37°C and incubated on a hot plate at 37°C with 5mls. of radioactive soak solution. The soak solution was prepared by the addition of ⁸⁶Rb to Krebs solution (Table 3.1) to a final concentration of 0.1μCi/ml.

In some experiments the media was replaced with fresh or conditioned medium containing 0.1μCi/ml. ⁸⁶Rb⁺ and the uptake was measured directly from this solution. After the incubation period, usually 10 min., the soak solution was removed by aspiration and the cultures were washed 4 times in ice cold Krebs. The cells were then removed from the growing surface by a 10 min. incubation, at 37°C, with 1ml. of a 0.25% trypsin solution (v/v in Ca⁺⁺ and Mg⁺⁺ free Earles basal salt solution). The trypsin was neutralised by the addition of 9 mls. of Krebs solution and any remaining cell clumps were dispersed by repeated syringing through a wide bore stainless steel needle. 1 ml. of the resulting suspension was added to 9.0 mls. of 'Isoton' counting fluid for the estimation of cell number and volume. 9.0 mls. were added to scintillation vials for Cherenkov counting.

The potassium concentration in Krebs was measured by flame photometry at the beginning of the experiment. By relating the counts per minute in the soak solution to K⁺ concentration it was possible, on measurement of the c.p.m. in the sample, to estimate the uptake in terms of moles per cell.

TABLE 3.1 Composition of Krebs solution.

Component	(mM)
NaCl	137
KCl	5.4
CaCl ₂	2.8
MgSO ₄ 7H ₂ O	1.2
NaH ₂ PO ₄	0.3
KH ₂ PO ₄	0.3
HCl	12
Tris base	14
Glucose	11
Calf serum	1% (v/v)
pH	7.36

Potassium free Krebs solution was prepared by omitting KCl and replacing KH₂PO₄ by NaH₂PO₄. Dialysed serum replaced normal serum.

Preparation of dialysis tubing

The manufacture of dialysis tubing involves the use of a polymeriser which often remains in the final product as an impurity (Stanton, personal communication).

Removal of these impurities was accomplished by the following procedure: the tubing was boiled for 5 min. in 0.1M NaOH, washed thoroughly in distilled water, boiled in 2% NaHCO₃ for 5 min. and washed thoroughly in distilled water before boiling in 3 changes of distilled water for a total of 15 min.

Preparation and concentration of conditioned medium

The cell cultures were grown for 2-3 days in normal E.M.D.M. (see Chapter I) after which time the media was decanted and the culture was washed x4 with serum containing or serum free medium. The fresh medium was added and the culture was equilibrated with a gaseous mixture of 95% air 5% CO₂ and incubated at 37°C for 12-18 hours. When the medium had become conditioned by this incubation, it was decanted and centrifuged at 100,000 x g for 1 hr. at 4°C in the J20 rotor of the Beckman J 21B centrifuge, in order to remove cell debris. The media was millipore filtered and used immediately or concentrated and stored at -20°C.

Concentration of the conditioned medium was achieved by dialysis against a concentrated solution of the high molecular weight carbohydrate, ficoll. The conditioned medium was decanted into dialysis tubing, prepared by the above method, and dialysed against a saturated solution of ficoll for 24 hrs. at 4°C with constant stirring. The

conditioned medium was then dialysed against 10mM Tris HCl (pH7.4) at 4°C for 24 hrs.

Ficoll which had been used for the concentration of conditioned medium was 'reconditioned' in the following manner: dialysis against 10mM Tris HCl pH7.4 at 4°C for 24 hrs; centrifugation at 100,000 x g (J20 rotor J21B centrifuge) for 3 hrs. at 4°C; concentration by rotary film evaporation and finally autoclaving at 30 lb/in² for 15 min. The 'reconditioned' ficoll was stored at -20°C in sterile containers until required.

Estimating the effect of conditioned medium

To determine the effect of conditioned medium on rubidium ion uptake, the normal growth medium was decanted and replaced by conditioned medium or fresh medium with or without serum and the cultures were incubated for 30 min. at 37°C. The media were decanted and the cells were washed x2 with normal Krebs solution and 5 mls. of soak solution was applied. The difference between the uptakes in Krebs after treatment with fresh and conditioned medium was taken as a measure of the conditioning effect. In some experiments ⁸⁶Rb uptake was measured directly from fresh and conditioned medium. In this case, the cultures were preincubated with fresh or conditioned medium for 30 min. and the uptake of ⁸⁶Rb was then measured in Krebs solution or in fresh or conditioned growth medium, as previously described.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed as

described by Kadlubowski (1976). The solutions used were as follows:-

- 1) 10. TEMED(w/v) in 99% ethanol.
- 2) 10% ammonium persulphate(w/v) in H_2O .
- 3) 5% acrylamide(w/v) 0.05% Bis.(w/v) in 50mM Tris/cl pH 7.4.
- 4) 50mM Tris/Cl pH 7.4.
- 5) 0.025% Coomassie brilliant blue (w/v) in 10% acetic acid (v/v), 20% methanol(v/v) and 70% H_2O .

Polyacrylamide was prepared by mixing 0.1 parts of solution 1 with 0.05 parts of solution 2 and 10 parts of solution 3. 1.1mls. portions of the mixture were added to glass tubes and overlayed with a few drops of solution 4 and allowed to polymerise for 30 min. The overlay was then replaced and after a further 12 hr. the gels were used. All gels were prerun with solution 4 for 30 min. at 6mA/tube on a Quickfit gel apparatus. Each reservoir was then refilled with 200 mls. of solution 4. A small volume of sample material was then added to the top of each gel and Bromophenol blue tracker dye was added to one tube. The gels were run for 5 min. at 2mA/tube and then at 6mA/tube until the tracker dye was almost at the bottom of the gel. The various protein bands were stained and fixed in position by soaking overnight in solution 5. The gels were then destained by washing in solution 5 minus the Coomassie blue.

RESULTS

The purpose of the present study was to establish the effects of cell density on the activity of the plasma membrane (Na+K)-ATPase. The effect of conditioned medium impinges on this study only in so far as it has been reported that conditioned medium exerts a stimulatory effect on the uptake of $^{86}\text{Rb}^+$ in various cell lines. If conditioned medium produces the above response, it would be particularly enlightening to investigate its effect on the (Na+K)-ATPase from cells of high density cultures.

Mouse cells are very insensitive to ouabain inhibition (Aiton 1976); indeed at high cell density ouabain has an apparently stimulatory effect on the activity of the $^{86}\text{Rb}^+$ uptake into transformed 3T3 cells (Brown 1976). It has been reported (Bonting 1971) that, at $1/300^{\text{th}}$ of the concentration required for inhibition in HeLa cells, ouabain has a stimulatory effect on the activity of the (Na+K)-ATPase. Moreover, Huxham (personal communication) has observed a stimulation of the sodium pump in these cells at low concentrations of ouabain. Thus it seemed that the results of Brown on the stimulation of the pump by high concentrations of ouabain at high cell density might be explained if the K_i for ouabain were increased in high density cultures of transformed 3T3 cells. However, at concentrations of 10^{-3}M ouabain the (Na+K)-ATPase activity of isolated plasma membrane was completely inhibited and at concentrations of ouabain as low as 0.01mM no stimulation of the enzyme was observed.

These observations are not incompatible since the effective concentration of ouabain at the surface of the intact cell might be reduced by steric hinderance.

In view of the difficulties associated with the use of ouabain in mouse cells it seemed better under the circumstances to measure the total rubidium flux rather than the ouabain sensitive component.

The uptake of ^{86}Rb in all the data to be reported is expressed in terms of total cell numbers rather than cell volume as has been the practice (Brown 1976). It would have been more valid to express the results in terms of cell surface area but there is as yet no procedure for obtaining a reliable estimate of this parameter in cultured cells (Aiton 1976). The cell surface area is only simply related to volume if the cells exist as perfect spheres. This is certainly not the case when cells are attached to petri dishes and though with trypsinisation the cells are reputed to be perfectly spherical, if some protein component of the medium were to bind more firmly under some circumstances, the apparent volume might alter markedly. Since the experiments were conducted on cell cultures of similar density ($1.0 \times 10^6/20\text{cm}^2$ in the case of 3T3 cells and $2.0 \times 10^6/20\text{cm}^2$ in the case of SV 3T3 cells), their initial volume and, it is hoped, surface area will not vary markedly between experiments.

Conditioned medium increases the rubidium uptake into normal and virus transformed 3T3 cells. The uptake into 3T3 cells was 40% greater after conditioned medium pretreatment

and the uptake into SV40 3T3 cells was increased by 42% (Fig. 3.1). These results are in good agreement with those of Brown et al but the effect which he observed was extremely variable (personal communication). This result tends to suggest that conditioning of the medium might not reconcile the observations on the rate of rubidium transport and activity of the (Na+K)-ATPase as the cell density increases.

The use of serum containing conditioned medium does not exclude the possibility that the cells, during their growth, remove from the serum component of the medium factor(s) which inhibit the sodium pump. However, the effect cannot be dismissed so simply. Fig. 3.2 shows that serum free conditioned medium also exerts a stimulatory effect on the rate of uptake of rubidium ions. There is a significant, two fold, increase in ^{86}Rb uptake after treatment with serum free conditioned medium. The effect of conditioned medium is, therefore, independent of the presence of serum, an observation which would facilitate future attempts to purify the factor(s) involved in the stimulation of the pump.

In none of these experiments could the effect of conditioned medium be attributed to perturbations of the cell-medium interface since, in all cases, the medium was decanted and replaced by either fresh or conditioned medium.

Attempts were made to establish the heat stability of the conditioning factor(s). Fig. 3.3 shows the effect of boiling fresh and conditioned medium prior to application to the cell cultures. These results show that boiling for up to 30 min. had no significant effect on pretreatment by either fresh or conditioned medium.

Fig. 3.1 The effect of pretreatment with fresh or conditioned medium on the uptake, measured from Krebs solution, of ^{86}Rb into 3T3 and SV40 3T3 cells.

C:- conditioned medium pretreatment

F:- fresh medium pretreatment

S:- SV40 3T3 cells

3:- 3T3 cells.

The uptake of ^{86}Rb from Krebs solution in both 3T3 and SV40 3T3 cells is 30% lower after fresh medium pretreatment than after conditioned medium. In SV40 3T3 cells, the uptake is 57 ± 5 and 40 ± 3 M/ 10^{15} cells/10min. respectively after conditioned and fresh medium pretreatment. In 3T3 cells, the influx is 46 ± 2 after conditioned and 32 ± 2 / 10^{15} cells/10min. after fresh medium pretreatment ($p=0.02$ in both cases).

The columns are the mean of 4 experiments performed in duplicate and the vertical bars represent the standard error of the mean.

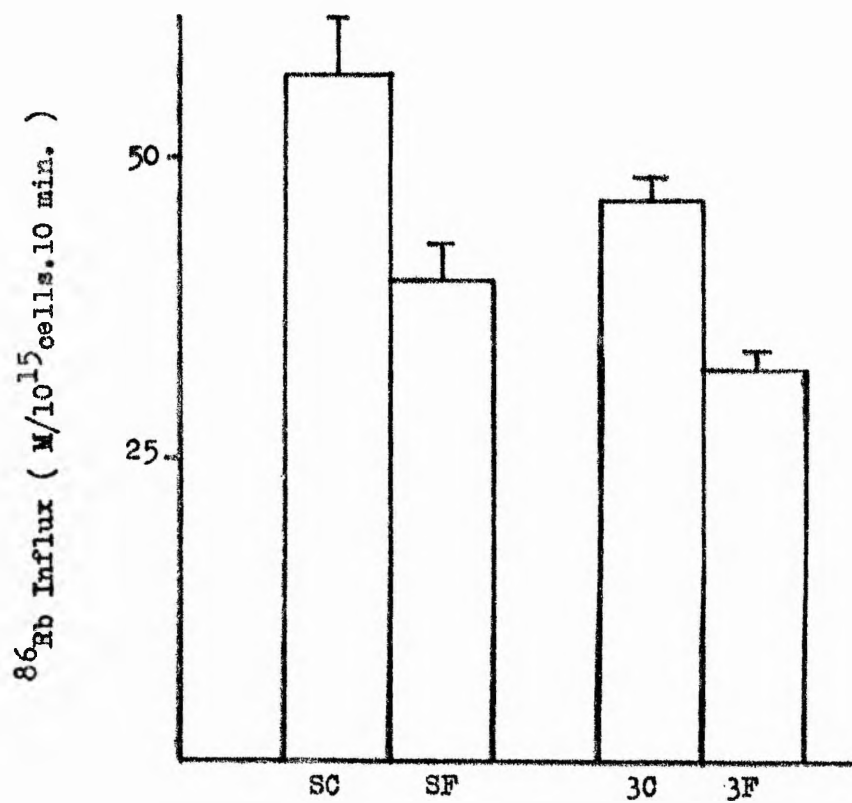


Fig. 3.1 The effect of pretreatment with fresh or conditioned media on the uptake of ^{86}Rb into 3T3 and SV₄₀3T3 cells.

Fig 3.2 The effect of serum-free and serum-containing conditioned medium on the uptake of ^{86}Rb into 3T3 cells.

C:- conditioned medium pretreatment

F:- fresh medium pretreatment

+S:- containing serum

-S:- without serum

The effect of conditioning is greater in the absence of serum. In this case the increase is two-fold (from 43 ± 3 to 88 ± 8 ; $p = 0.02$). There is no significant difference in the uptake after pretreatment with serum containing conditioned medium and the uptake after fresh medium without serum.

The columns are the mean of four duplicate observations and the vertical bars represent the standard error of the mean.

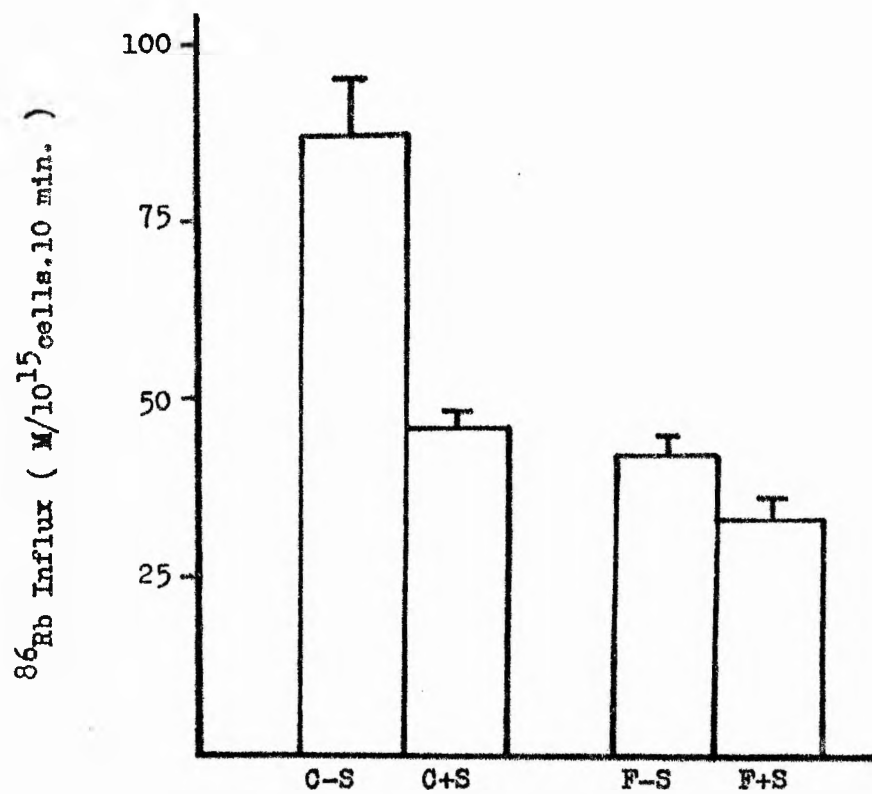


Fig. 3.2 A comparison of the effects of serum-free and serum containing fresh and conditioned media.

Fig 3.3 The effect of heat treated conditioned medium on the response of 3T3 cells.

C:- conditioned medium pretreatment

F:- fresh medium pretreatment

1:- boiled for 10 mins

2:- boiled for 20 mins

3:- boiled for 30 mins

Boiling the media for up to 30 mins did not significantly effect the subsequent uptake of ^{86}Rb from Krebs solution.

The columns are the mean of four duplicate observations and the vertical bars represent the standard error of the mean.

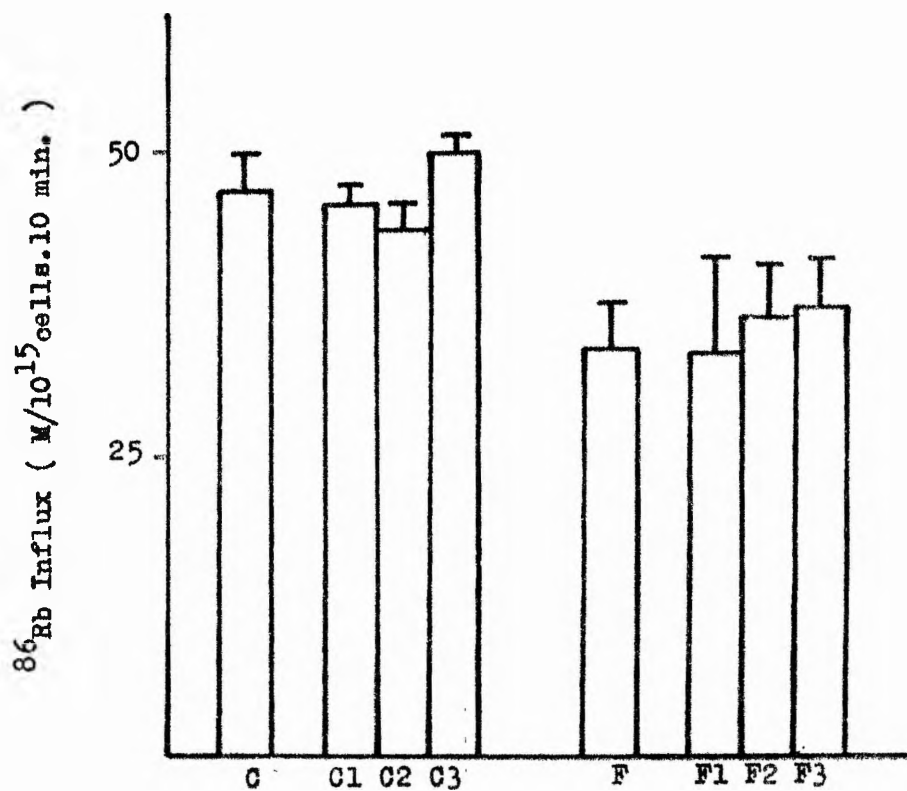


Fig. 3.3 The effect on subsequent ^{86}Rb influx of boiling fresh and conditioned medium.

The results of Brown et al (1975) on the effect of dialysis of the media against Krebs solution are confirmed in Fig. 3.4. The media were dialysed against Krebs at pH7.4 and at pH8.1 in an attempt to determine whether the factor(s) was more stable at alkaline pH. Conditioned media dialysed against Krebs at pH7.4 and pH8.1 were not significantly different in their effect. In both cases the subsequent uptakes of ^{86}Rb were around 50% of control values. Dialysis of fresh medium also reduced its effect on the subsequent uptake of ^{86}Rb from Krebs solution. The results in Figs. 3.3 and 3.4 suggest that although a preliminary removal of low molecular weight compounds by dialysis might not be possible, concentration of the medium by rotary evaporation is a distinct possibility in any isolation procedure.

Since the effects of conditioned medium on the subsequent uptake of ^{86}Rb from Krebs had been reported to be variable it was decided to attempt to measure the uptake of the nuclide directly from fresh and conditioned EMDM. Fig 3.5 shows that in fresh medium the uptake into 3T3 cells was significantly greater than the uptake from conditioned medium. However, the uptake from fresh EMDM and Krebs after pretreatment with fresh medium are not significantly different (Fig. 3.5 c.f. 3.1). This experiment suggests that the effect of changing the cells into Krebs solution produces an artificial increase in the influx of the nuclide.

This effect is not limited to normal cells. SV40 3T3 cells exhibit the same phenomenon (Fig. 3.6). After fresh EMDM pretreatment the ^{86}Rb uptake was the same in fresh EMDM

Fig 3.4 The effect of pretreatment with dialysed conditioned and fresh media on the subsequent uptake of ^{86}Rb into SV3T3 cells from Krebs solution.

C:- conditioned medium pretreatment

F:- fresh medium pretreatment

D1:- dialysed against Krebs pH 7.4

D2:- dialysed against Krebs pH 8.0

Dialysis of fresh and conditioned medium reduces the subsequent uptake from Krebs. The uptake after conditioned medium pretreatment is reduced 50% by dialysis (from 47 ± 4 to 23 ± 1.4 ; $p = 0.01$) and fresh medium by 18% (from 33 ± 3 to 27 ± 1 ; $p = 0.05$).

The columns represent the mean of four duplicate observations and the vertical bars represent the standard error of the mean.

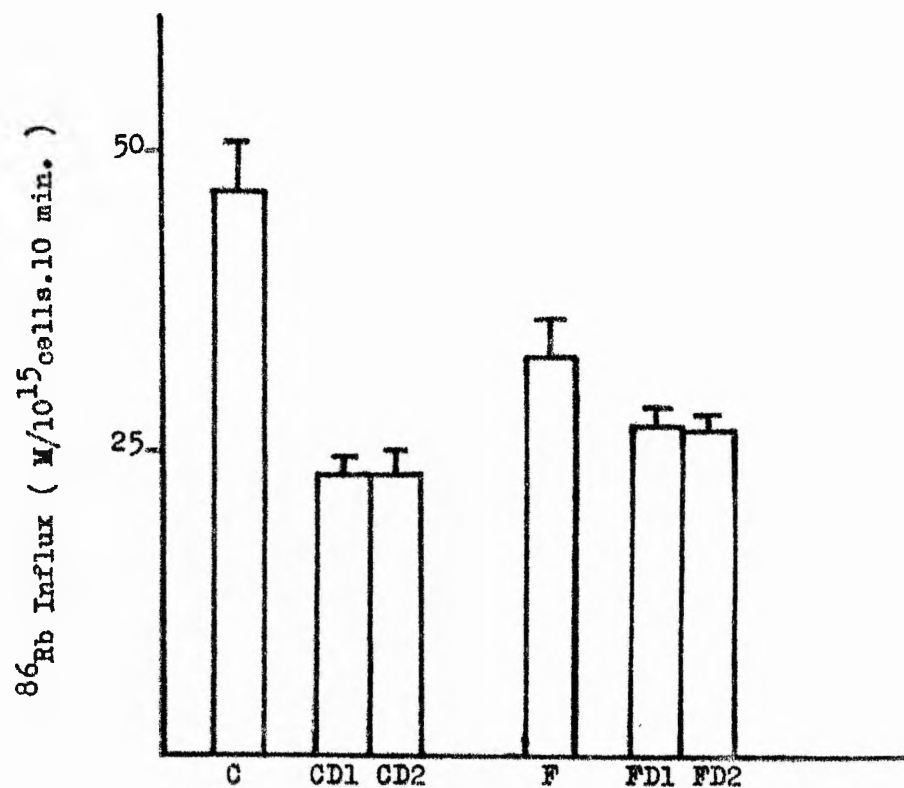


Fig. 3.4 The effect of dialysed fresh and conditioned media on the uptake of ^{86}Rb into 3T3 cells.

Fig. 3.5 The uptake of ^{86}Rb into 3T3 cells from fresh and conditioned growth media.

The uptake measured directly from fresh growth medium (FRESH) was 14% greater than that measured from conditioned growth medium (35 ± 1 c.f. 30 ± 1 M/ 10^{15} cells/10 min.; $p=0.05$)

The columns are the mean of 4 experiments and the vertical bars are the standard error of the mean.

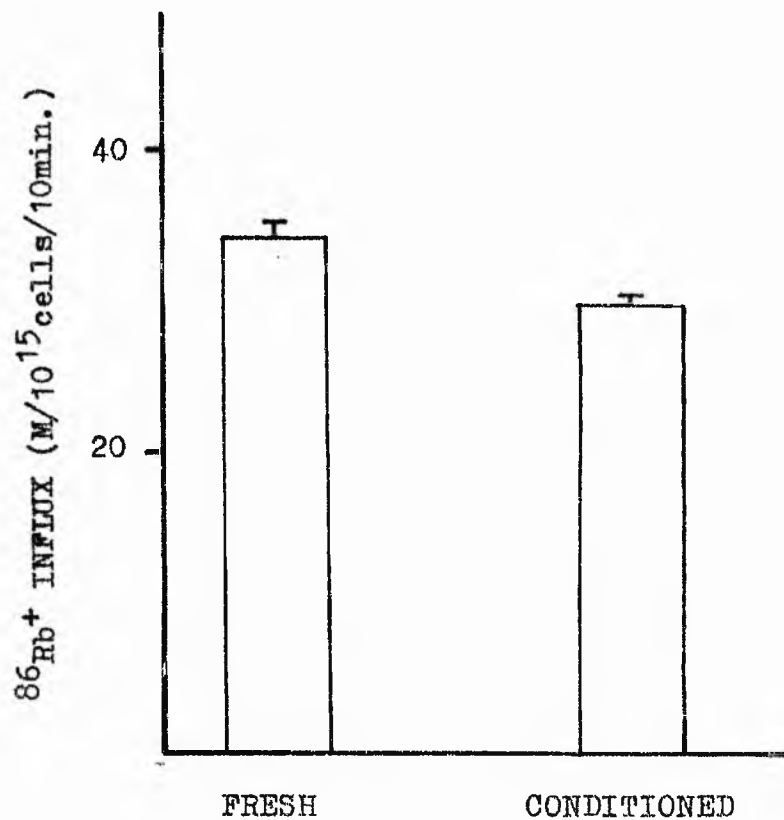


Fig. 3.5 The uptake of $^{86}\text{Rb}^+$ into 3T3 cells from fresh and conditioned growth medium.

Fig 3.6 The uptake of ^{86}Rb into SV3T3 cells measured directly from fresh and conditioned medium.

CK:- uptake from Krebs after pretreatment with conditioned medium

CG:- uptake from conditioned medium measured directly

FK:- uptake from Krebs after pretreatment with fresh medium

FG:- uptake from fresh medium measured directly

The uptake measured directly from fresh medium is not significantly different from the uptake in Krebs solution after fresh medium pretreatment. However, the uptake measured directly from conditioned medium is significantly less than in Krebs solution after conditioned medium pretreatment (57 ± 4 compared with 48 ± 1 ; $p = 0.02$). The latter value is also significantly less than the uptake from fresh growth medium.

Columns are the mean of four duplicate observations and the vertical bars represent the standard error of the mean.

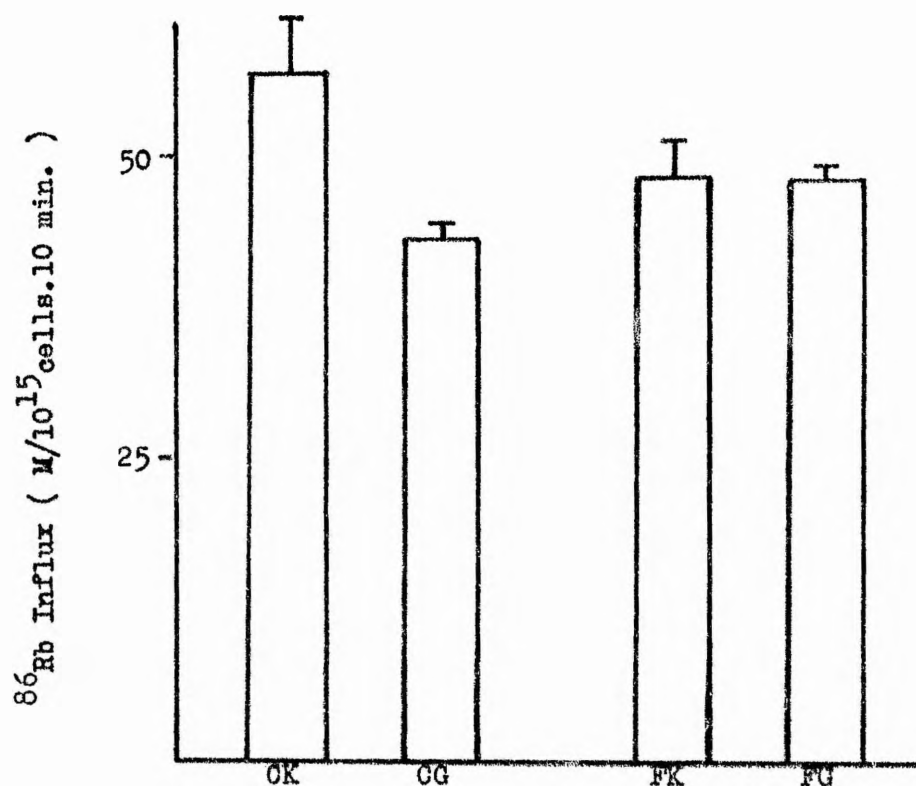


Fig. 3.6 A comparison of the ^{86}Rb uptake into $\text{SV}_{40}\text{T3}$ cells from fresh and conditioned growth media with the uptake from Krebs after preincubation in fresh or conditioned media.

and Krebs solution but the effect of conditioned medium depended on the conditions of measurement. If the effect of conditioned medium were measured in Krebs then the uptake was greater than after fresh medium pretreatment. However, if the uptake were measured directly from conditioned EMDM it was significantly less than that after fresh medium pretreatment. It was concluded that the increased uptake previously reported with conditioned medium was an effect of the transfer from conditioned medium to Krebs.

The results of preliminary experiments on varying the pH of the preincubation medium suggested that the conditioning effect might be due to an alteration in the hydrogen ion concentration of the growth medium. ²Belg² and Adragara (1974) had shown that the $^{86}\text{Rb}^+$ uptake into human erythrocytes was pH dependent and that the process showed a pH optimum of 7.4, and so it seemed that an alteration in pH might reduce the active flux causing the ion gradient to run down. On placing the cells in a Krebs soak solution, the optimum pH would be reestablished and the pumping activity would be increased by virtue of the higher intracellular Na^+ . However, the results of the pH experiments were so variable - sometimes an increase, sometimes a decrease, sometimes no change - that this particular investigation was not completed. In any case previous experiments had shown that conditioning per se did not increase the $^{86}\text{Rb}^+$ flux into these cultured cells.

Fresh medium, after 24 hrs. in culture was not significantly different from conditioned medium in its effect on ^{86}Rb uptake. However, the growth of 3T3 cells

in medium conditioned by 3T3 cells, was reduced compared to the growth of these cells in fresh medium. If serum free conditioned medium was made 5% with respect to serum, and added to cultures of 3T3 cells for 24 hrs., the number of cells in culture were found to be fewer than if the cells had been incubated in fresh medium containing 5% serum (Fig. 3.7). However, there was no significant difference between fresh and conditioned medium containing 2% serum.

The growth of 3T3 cells is reduced in medium conditioned by 3T3 cells. However, if the cells were incubated for varying times in medium conditioned by SV40 3T3 cells the number of cells per dish was greater than if the cultures had been incubated in fresh medium (Fig. 3.8). The stimulatory effect is not due to the removal of inhibitory substances from the serum, since, serum free medium conditioned by SV40 3T3 cells also stimulated growth. When serum free medium, previously conditioned by the growth of SV40 3T3 cells, was made 0.4% with respect to serum, the number of 3T3 cells in culture after a 72 hr. incubation was twice that of controls. This value was only slightly less than in fresh medium containing 2% serum (Fig. 3.9).

The stimulatory effect is not entirely lost on dialysis and concentration against ficoll (Fig. 3.10). Plate 3.1. shows polyacrylamide gel electrophoresis gels of concentrated conditioned medium from 3T3 and SV40 3T3 cells. The gel pattern is not complex and there is not much difference between the two cell lines. This observation seemed interesting but the stimulatory effect was small and since it was far removed from the main line of this research, it was not investigated further.

Fig 3.7 The growth of 3T3 cells in medium conditioned by 3T3 cells.

F:- fresh E.M.D.M.(Eagle's medium Dulbecco's modification)

C:- conditioned E.M.D.M.

5%:- containing 5% serum

2%:- containing 2% serum

Serum-free medium was conditioned by supporting the growth of log phase cultures of 3T3 cells for 24 hours. The medium was then made 5% or 2% with respect to serum and added to the log phase cultures of 3T3 cells. The cell numbers were measured 24 hours later and expressed in terms as a % of the figure obtained with fresh medium containing 5% serum.

The number of cells in cultures treated with 5% conditioned medium was significantly less than ($p = 0.05$) in those treated with 5% fresh medium. However, there was no significant difference in numbers from cultures treated with 2% fresh and conditioned medium but these values are significantly less than those cultures treated with 5% fresh medium.

The columns represent the mean of two duplicate observations and the vertical bars represent the standard error of the mean.

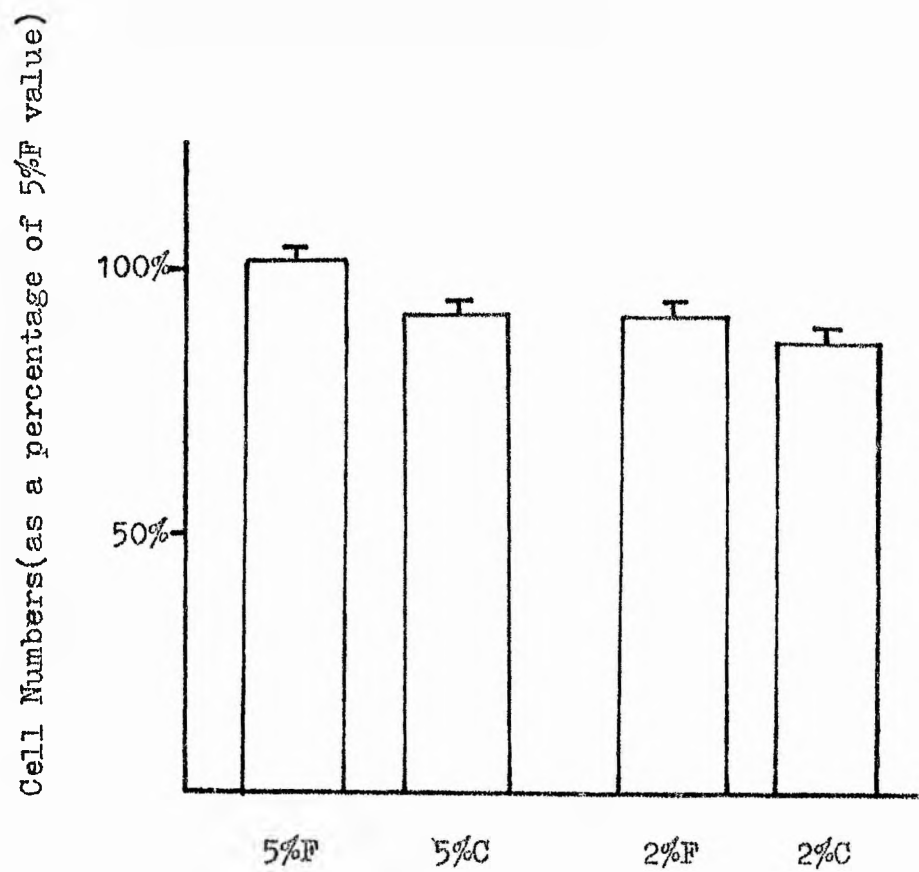


Fig. 3.7 The growth of 3T3 cells in medium conditioned by 3T3 cells

Fig. 3.8 The growth of 3T3 cells in medium conditioned by SV40 3T3 cells.

- :- cells grown in medium containing 10%(v/v) serum which had been conditioned by the growth of SV40 3T3 cells.
- :- cells grown in fresh medium containing 10%(v/v) calf serum.
- :- cells grown in fresh medium containing 0.4%(v/v) calf serum

The number of cells in culture, after 48Hrs., was significantly less in fresh than in conditioned medium ($p=0.05$ by the students T test.)

Cultures were seeded initially in fresh medium containing 10%(v/v) calf serum, and, after 24Hrs., the medium was discarded and replaced by fresh or conditioned medium containing 10%(v/v) calf serum or fresh medium containing 0.4%(v/v) calf serum. The cultures were incubated for various times, the cell numbers estimated and compared to initial levels. ($n=3 \pm \text{SEM}$)

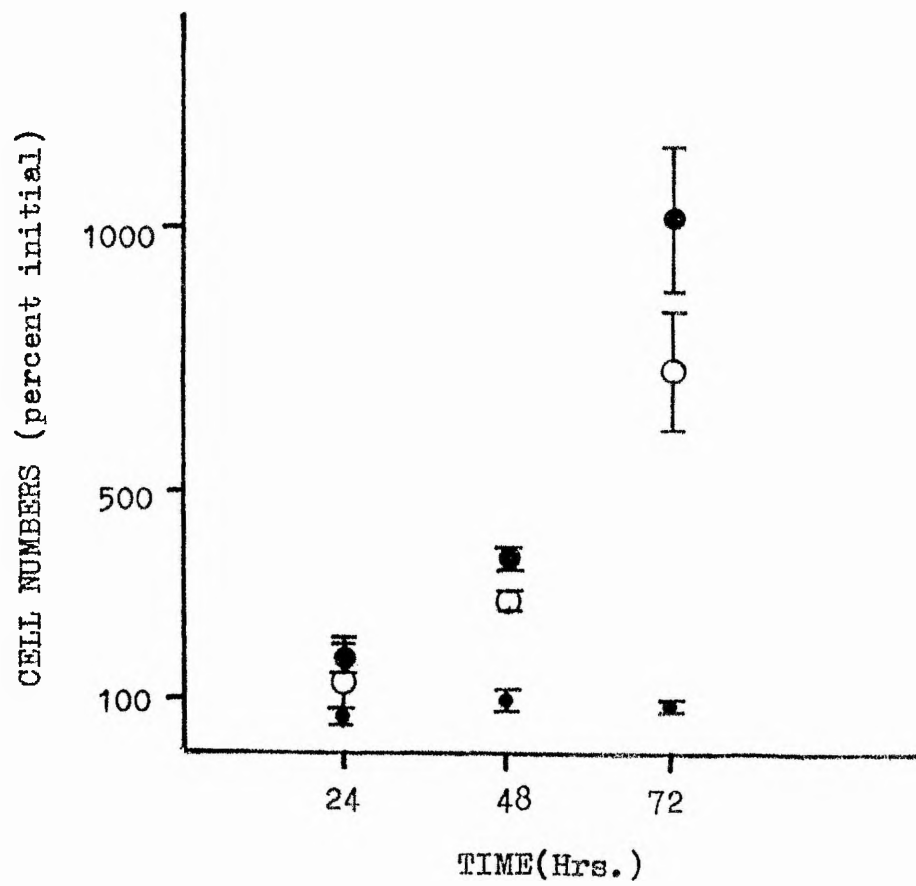


Fig. 3.8 Growth of 3T3 cells in medium conditioned by SV40 3T3 cells.

Fig. 3.9 The growth of 3T3 cells in medium conditioned by SV40 3T3 cells.

Cond.:— serum free conditioned medium adjusted to 0.4%(v/v) with respect to calf serum.

2%F :— fresh medium adjusted to 2%(v/v) with respect to calf serum

5%F :— fresh medium adjusted to 5%(v/v) with respect to calf serum.

After 72 Hrs. the number of cells from cultures incubated in the presence of conditioned medium was twice that of initial levels but less than that in fresh medium containing 2 or 5% calf serum($p < 0.05$ in both cases).

The experimental procedure was similar to that described in the legend to Fig. 3.8. ($n=3 \pm \text{SEM}$).

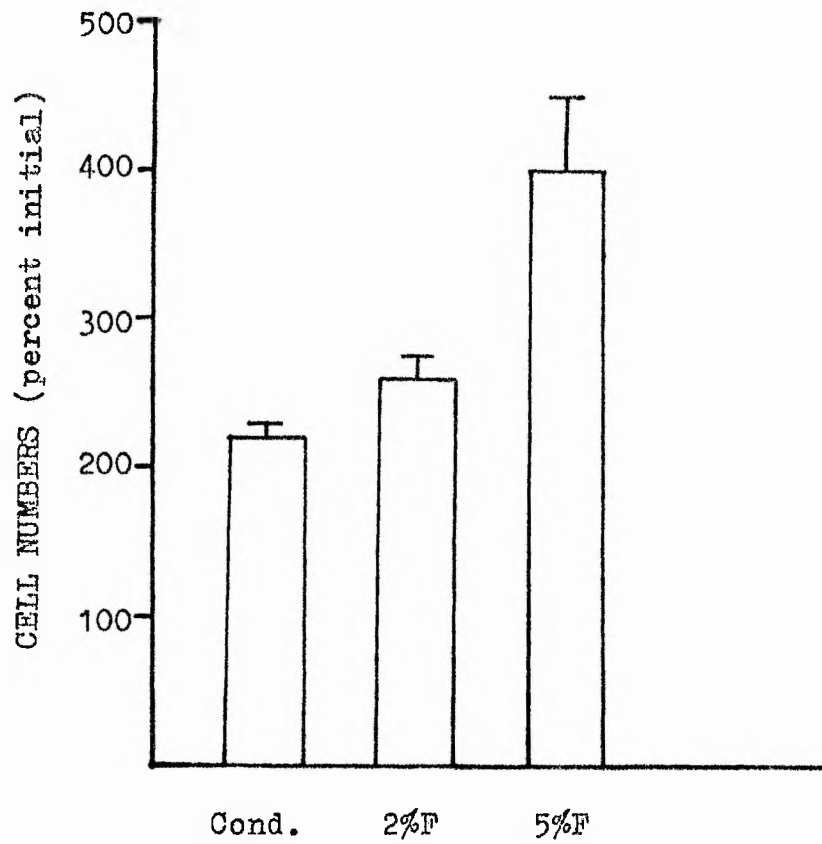


Fig. 3.9 Growth of 3T3 cells in medium conditioned by SV40 3T3 cells.

Fig. 3.10 The growth of 3T3 cells in medium conditioned by SV40 3T3 cells.

C.Cond :- concentrated serum free conditioned medium added to fresh medium and adjusted to 0.4% (v/v) with respect to serum.

Cond. :- serum free conditioned medium adjusted to 0.4%(v/v) with respect to calf serum.

5%Fresh:- fresh medium containing 5%(v/v) calf serum

The stimulatory effect of conditioned medium is not significantly reduced by concentration against a saturated solution of Ficoll. The number of cells present after 72Hrs. in culture was not significantly different in conditioned medium or fresh medium to which had been added, concentrated conditioned medium. In both cases the number of cells was twice that of controls.

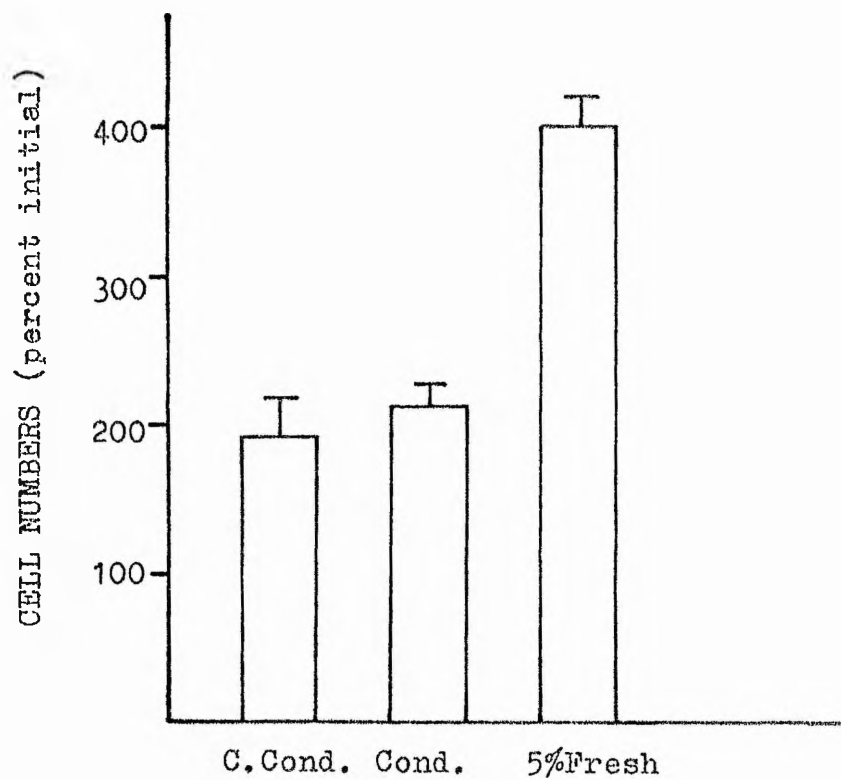


Fig. 3.10 The growth of 3T3 cells in medium conditioned by SV40 3T3 cells.

PLATE 3.1

Polyacrylamide gel electrophoresis of conditioned medium.

S:- SV40 3T3 cell conditioned medium

3:- 3T3 cell conditioned medium.

The gels were prepared and run as described in the METHODS section.

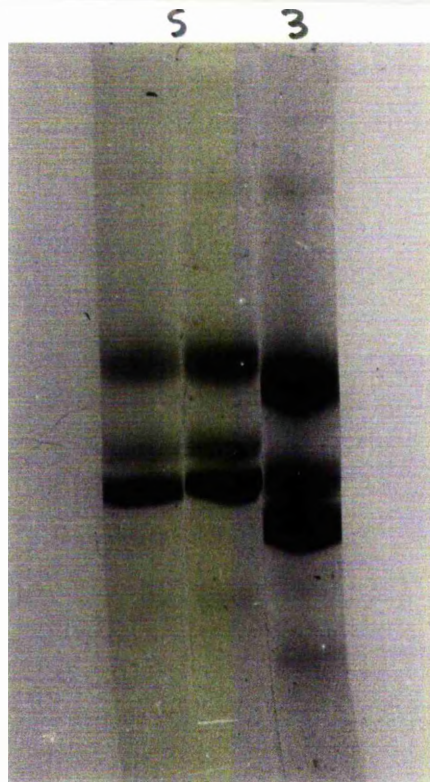


PLATE 3.1 Polyacrylamide gel electrophoresis of concentrated serum-free medium conditioned by 3T3 or SV40 3T3 cells.

DISCUSSION

The series of experiments described in this section were performed in order to determine whether conditioning of the medium, during the growth of cells, stimulated the uptake of rubidium ions. The results show that this is not the case; the apparently stimulatory effect of conditioned medium is only observed when the uptake of $^{86}\text{Rb}^+$ is measured from Krebs solution, and the uptake measured directly from conditioned medium is reduced compared to that from fresh growth medium. Therefore, it appears that the reported stimulatory effect of conditioned medium is induced by the transfer of cells from conditioned medium to Krebs solution.

It has been reported (Holley and Kiernan 1971), that growing cultures of 3T3 cells remove stimulators of growth from their growth medium. This observation appeared to hold in the present investigation but SV40 3T3 cells released substances capable of promoting the growth of normal 3T3 cells. The origin of these substances is unlikely to be serum factors, since SV40 3T3 cells do not remove factors necessary for the growth of 3T3 cells. As Pasternak (1976) remarks, 'The fact that cultured cells are able to synthesise such components is not as surprising as might appear at first sight' the cells, after all, before transformation, contain a complete genetic complement. Pasternak suggests that the inability of normal cells to produce growth promoting substances might reflect no more than a reduced leakiness of their plasma membranes. However, the results reported here suggest that

the 3T3 cells release growth inhibitors.

Preliminary experiments, not reported here, suggested that the protein components shown by gel electrophoresis could be separated by chromatography on a DEAE-cellulose column. However, the effect of conditioning on growth was small and since the phenomenon was not directly related to the mainstream of the present investigation, the subject was not pursued further.

CHAPTER IV

CELL POPULATION DENSITY ASSOCIATED
ALTERATIONS IN THE PROPERTIES OF
CULTURED FIBROBLASTS

SUMMARY

1. The effect of cell population density on the activity of 'cell surface' and 'intracellular' enzymes in 3T3, Py3T3, SV 40 3T3 and HeLa cells was examined. Concanavalin A binding was estimated in 3T3, SV40 3T3 and HeLa cells and ouabain binding was measured in HeLa cells.
2. When SV40 3T3 and Py3T3 cells were grown in medium containing 10% newborn calf serum, the activity of the 'cell surface' enzymes decreased with increasing population density. The (Na+K)-ATPase activities decreased sharply at densities of 5×10^4 cells/cm² and the 5'-nucleotidase activity decreased gradually with density but both activities were reduced to the same extent as the density exceeded 20×10^4 cells/cm². In contrast to the virus transformed cells, the activity of the 'cell surface' enzymes of 3T3 cells increased as the population density increased above 5×10^4 cells/cm².
3. The decrease in the activity of the (Na+K)-ATPase with increasing density could not be attributed to an increase in the apparent Km or to the acquisition of a non-linear timecourse and it was concluded that the decrease was due to a reduction in the Vmax for the reaction.

4. Treatment of SV40 3T3 cells homogenates with 2-deoxycholate did not increase the activity of the (Na+K)-ATPase or 5'-nucleotidase tending to suggest that the enzymes do not become cryptic as the culture density increases.
5. Pretreatment of cultures of SV40 3T3 cells with media containing 0% or 20% newborn calf serum decreased and increased, respectively, the activity of the 'cell surface' enzymes. However, the activities of all other enzymes investigated were increased in media containing 20% serum and decreased in media containing 0% serum. It was concluded that the effect of serum was non specific.
6. When cultures of SV40 3T3 cells at densities of $20-30 \times 10^4$ cells/cm² were treated with trypsin and incubated for 18hr. in fresh medium containing 10% newborn calf serum, there was a specific increase in the (Na+K)-ATPase activity. The increase required trypsinisation prior to the addition of fresh medium and did not occur when trypsinised cultures were incubated with conditioned medium. It was concluded that the effects of trypsin and fresh serum were synergistic.
7. The 'cell surface' enzymes of HeLa cells show a similar relationship to culture density as those of Py and

SV40 3T3 cells. In HeLa cells, the decrease in the activity of 5'-nucleotidase was associated with a decrease in the V_{max} for the reaction.

8. In HeLa cells the specific ouabain binding also decreased with population density. However, the size of the reduction and the density at which it occurred were not the same as for the (Na+K)-ATPase activity. It was concluded that the one need not be a linear function of the other.
9. The specific Concanavalin A binding decreased with density in HeLa, SV40 3T3 and 3T3 cells. In HeLa cells there was a 25% decrease in Concanavalin A binding as the culture density increased from $3-6 \times 10^4$ cells/cm². This corresponds well with the 24% decrease in 5'-nucleotidase activity observed over the same density range.
10. In SV40 3T3 cultures there was also a decrease in the specific Concanavalin A binding as the density increased. In this case the reduction in binding did not appear to be related to the decrease in the activity of the 5'-nucleotidase.
11. Although the cell protein and volume of all cell lines examined exhibited a decreasing trend with increasing density, the two factors did not appear to be linearly

related. It was suggested that, if the loss of protein occurred from the cell surface, it need not be associated with a reduction in the intracellular osmotic pressure.

12. The 'intracellular enzymes' of SV40 3T3 and Py3T3 cells grown in newborn calf serum were affected in similar ways by increasing population densities. Acid phosphatase activities increased and rotenone insensitive NADH-ferri-cyanide reductase activities decreased.
13. The activity of the 'intracellular enzymes' of 3T3 cells grown in 10% newborn calf serum were affected in the same way as the 'cell surface' enzymes. All of the 3T3 cell enzymes investigated increased as the cell population density increased.
14. In HeLa cells, increasing culture population density was associated with an increase in the activity of the rotenone insensitive NADH-ferricyanide reductase while the other enzymes were not significantly affected.
15. The effect of culture density was found to be dependent on the type of serum with which the growth media were supplemented. In 3T3 cells grown in medium containing 10% foetal calf serum, the activities of all of the enzymes measured, with the exception of the acid phosphatase, were observed to decrease as the culture density increased. When SV40 3T3 cells were grown in

medium containing 10% foetal calf serum, the activities of acid phosphatase and rotenone insensitive NADH-ferri-cyanide reductase altered in a manner similar to that seen in newborn calf serum, while the other enzymes appeared to be independent of population density.

16. It was concluded that the activities of the 'cell surface' and 'intracellular' enzymes were related, not only to cell population density, but also to the type of serum with which the growth medium had been supplemented.

INTRODUCTION

It has been known for some time that the properties of normal and transformed cells differ. At the multicellular level the rate of movement of transformed cells is greater than normal and the normal 'contact inhibition of movement' (Abercrombie 1954) is lost. Unlike normal cells, tumour cells, when they come into contact, do not adhere and their movement is not impeded. Contact inhibition of movement is usually associated with a reduction in the rate of cell division - which has come to be known as contact inhibition of growth - or 't̄ropoinhibition' (Dulbecco 1970). T̄ropoinhibition occurs at a density characteristic of the particular cell line e.g. 3T3 cells undergo t̄ropoinhibition at $10\text{--}20 \times 10^4 \text{ cells/cm}^2$ in calf serum while Baby Hamster Kidney (BHK) cells grow to much higher densities ($30 \times 10^4 \text{ cells/cm}^2$).

Several theories on the mechanism of t̄ropoinhibition have been briefly discussed in the general introduction. Although it seems unlikely that any single theory may explain so complex a phenomenon, it is clear that during this phase of culture growth there are alterations in some of the properties of the cell surface membrane.

Glucose uptake is probably the transport phenomenon which has been most extensively studied with respect to transformation and t̄ropoinhibition. Since glucose is metabolised, the analogues of glucose, 2-deoxy glucose (2-DOG) and 3-O-Methyl glucose (3-OMeG), are usually used in these investigations. Depending on conditions, glucose transport across

the membrane may or may not be rate limiting. Once glucose has entered the cell it is rapidly phosphorylated to Glucose-6-phosphate. The cell surface is impermeable to this compound and trapping occurs. The enzyme responsible for the formation of glucose-6-phosphate, hexokinase, is inhibited by its product so that the amount of trapping which occurs depends on various biochemical parameters within the cell. A similar problem is also encountered with 2-DOG. This substance acts as a substrate for the hexokinase and is trapped in the same way as glucose itself; its one advantage is that its metabolism does not proceed further than 2DOG-6-phosphate. The problem of trapping which occurs with 2-DOG is well recognised and attempts have been made to overcome this by estimating the activity of hexokinase under the various experimental conditions. However, unless the exact cellular concentration of G-6-P is reproduced in the test tube these measurements are of little relevance. 3-O-Methyl glucose on the other hand does not act as a substrate for hexokinase and its use as a glucose analogue might be preferred, if it were not for the fact that there is some doubt as to whether it is transported by the same mechanism as glucose (Romano 1977).

The uptake data are usually analysed in accordance with Michaelis-Menten principles. The V_{\max} is said to be a measure of the number of transport sites and the K_m apparent is supposed to be a measure of the affinity of the carrier for its substrate. These parameters for the reaction:



$$\text{are } K_m = \frac{K_2 + K_3}{K_1 + K_4} \quad \text{and } V_{\max} = K_3 E_t$$

where E_t is the total number of transport sites. It can be seen from these values that the affinity constant for substrate binding $\frac{K_2}{K_1}$ only approaches the Michaelis constant

when the $K_3 \ll K_2$ and $K_4 \rightarrow \infty$ and that the V_{\max} only yields a linear function of the number of transport sites when K_3 does not alter from one condition to the other. The first condition is only met when the transport of substrate across the membrane and release at the other side is slower than the rate of binding to the carrier.

Bearing in mind these reservations, there appears to be a mass of information to suggest that, after transformation by a virus or a chemical, there is an increase in the rate of transport of various metabolites into the cell. When normal cells become contact inhibited the rate of transport decreases, while transport in transformed cells is not affected by density.

Sefton and Rubin (1971) showed that the uptake of 2-DOG into chick embryo fibroblasts (CEF) cells decreased 13 fold when the cells became quiescent (ceased to divide). Weber (1973) observed a 6 fold decrease under these conditions and suggested that it was due to a 3 fold decrease in V_{\max} which was not associated with an alteration in the K_m for the reaction. According to Kletzein and Perdue (1974) the alteration in glucose transport must be due to a change in the number of carrier molecules, since the activity of the hexokinase did not significantly alter. These general findings seem to apply equally well to other cell lines which

exhibit ~~t~~ropoinhibition. In BALB/c 3T3 cells the uptake of 2-DOG was reduced 6-10 fold as the cell density increased from 10^3 to 2×10^4 cells/cm² (Bose and Zlotnick 1973). This reduction was associated with a 7 fold decrease in the Vmax of the system, while the Km did not appear to alter.

Transformation itself appears to bring about an increase in the rate of 2-DOG uptake. Plageman (1973) found that transformation of mouse cells by murine leukemia virus induced an increase in the rate of 2-DOG uptake by virtue of an increase in the Vmax rather than a change in the apparent Km. A similar situation is found after transformation of CEF cells by Rous Sarcoma virus (Kletzein and Perdue 1974). When normal cells are infected with a virus mutant which is temperature sensitive, a 2-3 fold increase in the uptake of 2-DOG may be observed at the permissive temperatures (Kletzein and Perdue 1974(c) ; Martin et al 1971). When CEF cells are infected with temperature sensitive mutants of Rous Sarcoma virus only the translation stage in protein synthesis is required for the increase in activity (Martin et al 1971; Kawai and Hanafusa 1971; Kletzein and Perdue 1974(c)). Not only does viral transformation appear to increase 2-DOG transport but it appears that transformed cells, presumably by virtue of their loss of contact inhibition, do not exhibit a decrease in the rate of 2-DOG uptake at high cell densities (Plageman 1973; Bose and Zlotnick 1973; Oshiro and Di Paulo 1974).

Transformed cells which have regained ~~t~~ropoinhibition are said to have reverted. Reverted cells, on quiescence,

have a reduced uptake of 2-DOG, which seems to be related to a 5 fold decrease in V_{max} with a 2.5 fold increase in K_m (Schultz and Culp 1973).

The weight of evidence, therefore, suggests that the rate of uptake of 2-DOG and therefore glucose is related to transformation and density, and is mediated by alterations in V_{max} rather than K_m . However, tumour cells have a higher rate of glycolysis than normal cells (Paul et al 1966). In addition, contrary to the reports of Kletzein and Perdue (1974) who maintain that hexokinase activity does not alter in relation to 2-DOG uptake, Colby and Romano (1973 and 1974) have observed that the increase in 2-DOG transport associated with viral transformation of 3T3 cells is related to increased phosphorylation. It was shown that while the initial rates of uptake of 2-DOG in SV40 3T3 cells were 3 fold greater than in 3T3 cells, the intracellular free glucose was the same in both cases. Furthermore, Romano (1977) has defended the view that transformation does not lead to an increase in glucose transport per se. He suggests that, due to the problem of phosphorylation, the uptake of 2-deoxyglucose does not reflect the capacity of the cell membrane to transport glucose.

In addition, there are doubts concerning the use of 3-OMeG as an analogue for glucose. The former is taken up so rapidly that the initial rates are difficult to establish and there is a large component of simple diffusion even at very low concentrations of 3-OMeG. The K_i for inhibition of 2-DOG uptake by 3-OMeG is ten fold greater than

the K_i for inhibition by glucose and the K_i for inhibition of 3-OMeG uptake by glucose is an order of magnitude higher than the K_i for inhibition of 2-DOG uptake. The situation is therefore complex and 3-OMeG uptake may even be independent of the glucose transport system or the hexokinase activity may be rate limiting.

Transport of some amino acids is reputed to be affected in much the same way as 2-DOG when normal cells are transformed by virus, contact inhibited or serum stimulated (See Brown 1976 for a review.) Tramacere et al (1977) have suggested that, in chick embryo fibroblasts at least, those amino acids whose transport is affected in this manner are transported by the A mediated or sodium dependent system (Christensen 1967). That is, the transport of these amino acids, like that of glucose is dependent on the sodium ion gradient across the plasma membrane.

The uptake of inorganic phosphate is also dependent on the sodium ion gradient. When the transport of inorganic phosphate is considered under the various conditions of transformation and ~~topo~~inhibition, it appears to vary in a manner similar to 2-DOG. When cell growth becomes arrested in the confluent state, the uptake of inorganic phosphate is reduced (Weber and Eldin 1971); no such decrease is observed in transformed cells. The addition of fresh serum re-stimulates both division and phosphate uptake (de Asua et al 1974). Furthermore, when inorganic phosphate is removed from the growth medium 3T3 cells become arrested in the G_1 phase of the cell cycle (Kamely 1977) and when inorganic phosphate is readded, the cells resume division. However,

it appears that the rapid increase in phosphate transport following the addition of serum to quiescent fibroblasts is not a necessary event for the initiation of cell proliferation (Greenberg et al 1977).

In view of the evidence, albeit controversial, for alterations in sodium dependent uptake systems associated with events which alter the growth of the cell, it is surprising that there has been, until recently, little interest in the effects of such events on the cells sodium pumping activity. Perhaps the main stumbling block to such investigations is the relative insensitivity to ouabain of those cells normally used for such investigations. Mouse cells require concentrations of around 10^{-3} M ouabain whereas HeLa cells require only 2×10^{-7} M for complete inhibition of the sodium pump.

Kimelberg and Mayhew (1976) have reported that the ouabain sensitive $^{86}\text{Rb}^+$ influx into SV40 3T3 cells, measured over a 5 min. period is 4.5 fold greater than in 3T3 cells. Banerjee et al (1977), on the other hand, report a decrease in the ouabain sensitive ^{86}Rb flux and ^3H ouabain binding of CEF cells after oncogenic transformation by Rous sarcoma virus.

Several authors have attempted to relate the activity of the (Na+K)-ATPase to the ouabain sensitive ^{86}Rb flux. The perils of this procedure are best illustrated by the work of Lelievre et al. Using MOPC 173 derived cell lines one of which, ME2, exhibited contact inhibition while the other, MF2, did not, they showed that as the density of contact inhibited cells increased from 4×10^4 cells/cm² to

8×10^4 cells/cm², the activities of the (Na+K)-ATPase and 5'-nucleotidase were reduced to 1% of previous levels. At densities from $5-20 \times 10^4$ cells/cm² the activities of these enzymes in the noncontact inhibited cells were not affected (Lelievre and Paraf 1973). In a later publication from the same group (Ducouret-Prigent et al 1975) it was reported that, although the (Na+K)-ATPase activity was reduced to levels as low as 5% in contact inhibited cells, the ouabain sensitive ⁸⁶Rb transport was not significantly altered.

Little agreement has been reached on the effects of viral transformation on the activity of the (Na+K)-ATPase. Kimelberg and Mayhew (1976) have suggested that the reported increase in the uptake of ⁸⁶Rb into 3T3 cells on transformation by SV40 virus is associated with a 2.5 fold increase in the Vmax of the enzyme. Ellgisen et al (1974) maintained that there was little difference in the activity of the enzyme in 3T3 and SV40 3T3 cells at low culture densities, while at high culture densities the 3T3 cells activity was reduced and the SV40 3T3 cell activity was not affected. Yoshikawa-Fudaka and Najima (1972) on the other hand, report that the activity of the SV40 3T3 cell enzyme, at culture densities of less than 5×10^4 cells/cm² is 75% of that of 3T3 cells. Graham (1972) reported that the (Na+K)-ATPase activity of plasma membranes from virus transformed BHK cells was lower than normal, while Sheinin and Onodera (1972) have observed that the (Na+K)-ATPase activity of plasma membrane isolated from virus transformed 3T3 cell lines may be greater or less than that of normal cells depending on the virus used.

The divergence of opinion might be explained in terms of the growth conditions used for the different investigations. Kimelberg and Mayhew used media containing 10% foetal calf serum, while Yoshikawa-Fudaka and Najima and Graham used media containing 10% calf serum. It might also be explained in terms of an alteration in activity associated with adaptation to growth in calf serum. Tupper (1977) has shown that after 50 passages in calf serum the active uptake of ^{86}Rb in 3T3 cells rose to levels greater than that of SV40 3T3 cells, while previously the level had been lower.

The activity of the (Na+K)-ATPase of normal 3T3 cells appears to be reduced on confluency. Elligsen et al (1974) has reported such a decrease which occurred dramatically as the culture density exceeded $5 \times 10^4 \text{ cells/cm}^2$. Coincidentally, this density is approximately the same as Lelievre reported to be the critical density for the decrease of (Na+K)-ATPase in ME2 cells. Neither author observed an alteration in transformed cells, although Lelievre's lowest density for MF2 cells was $5 \times 10^4 \text{ cells/cm}^2$.

The decrease in activity associated with increasing culture density may be reversed by the addition of fresh serum (Elligsen et al 1974). The activity of serum in this respect is not prevented by cycloheximide (Lever et al 1976). The increase in activity is associated with a resumption of growth and when the increase is prevented by the addition of ouabain the 3T3 cells do not enter the S phase (Tupper et al 1977). However, if ouabain is removed from the cells within 6hr. of serum stimulation the increase in transport occurs followed by the entry of the cells into the S phase of the

cell cycle. From this evidence it would appear that the activity of the sodium pump might be related to cell growth, but it has been reported recently (Lelievre et al 1977) that in these cells ouabain brings about an increase in the levels of cyclic AMP. If this were the case the inhibition of growth may be due to an increase in cyclic AMP levels.

MATERIALS

^3H Concanavalin A (con A; 50 $\mu\text{Ci}/\text{ml}$) and ^3H ouabain were obtained from the Radiochemical Centre (Amersham). 'Scintol 2' and triton-X100 (p-isooctylphenoxypolyethoxyethanol) were obtained from Koch-Light Laboratories Ltd. Con A was obtained from the British Drug House and all other reagents were of the analar grade.

Scintillation fluid was prepared by mixing 50mls of 'Scintol 2' with 425mls of triton-X100 and 425mls of Toluene (Peng 1977).

METHODS

Cell culture was performed as described in Chapter I. The cultures were seeded with different cell numbers at day zero and harvested 3 days later for experimentation. The cultures were prepared in this manner to determine the effects of cell population density on various cellular functions. Horvat and Acs (1974) suggest that when cells are subcultured from high to low density, it takes at least 2 days for the activity of the enzymes to alter from the high density values and at day 3 these alterations are maximal. In addition it is recommended (Flow Laboratories Ltd.) that the cultures be supplied with fresh growth media at 3 day intervals. If serum had had any direct effect on the levels of any of the enzymes measured, then it would have been less informative to make the observations after medium renewal. After 3 days in culture the cells were removed from the growing surface with the aid of a rubber policeman and syringed to disruption. Duplicate cultures were trypsinised and used for the estimation of cell number and volume.

Enzyme assays

Enzyme assays, with the exception of the exo-ATPase, were performed as outlined in Chapter I. The exo-ATPase activity was estimated by measuring the hydrolysis of 0.8mM MgATP^{2-} by whole cells incubated in 1.6mls. of Krebs solution (Table 3.1) at 35°C for 30 min. The integrity of the cells used for exo-ATPase estimation was examined by Nigrosin staining (Paul 1970).

Deoxycholate treatment

The cell homogenates were incubated with known quantities of 2-deoxycholate in the (Na+K)-ATPase or 5'-nucleotidase incubation media in the absence of substrate for 2 min. at room temperature. The substrate was then added, the mixture was incubated at 35°C and the enzyme activities were measured in the usual way.

Medium replacement

Cultures were grown in Roux bottles for 3 days as previously described (Chapter I) to a density of $20-30 \times 10^4$ cells/cm². The growth media were discarded, the cultures were washed with fresh medium containing 0, 10 or 20% newborn calf serum and incubated in one of these media for 18hr. at 37°C. The cells were removed from the growing surface, the various enzyme measurements were performed and the activity obtained was related to that observed in untreated cultures.

Trypsin treatment

The cultures were grown for 3 days to a density of $20-30 \times 10^4$ cells/cm² in medium containing 10% newborn calf serum. Several cultures were treated with 5 mls. of trypsin solution (0.025% v/v in Mg²⁺/Ca²⁺-free Earles Basal Salt Solution for 15 min. at 37°C) and incubated for 18hr. in either fresh or conditioned medium containing 10% newborn calf serum. For controls, 5 mls. of the trypsin solution were added to otherwise undisturbed cultures. After 18hrs.

incubation the cells were removed from the growing surface and their enzyme activities were compared to untreated controls.

Ouabain binding

Ouabain binding was performed as described by Aiton (1976). The specific ouabain binding was taken as the difference in ^3H ouabain bound in the presence and absence of 15mM K^+ . The Petri dish cultures were incubated at 37°C on thermostatically controlled bench incubators with Krebs solution containing $2 \times 10^{-7}\text{M}$ ouabain, ^3H ouabain (0.1 $\mu\text{Ci/ml.}$) and 0mM or 15mM K^+ . After 45 min. incubation the radioactive soak solution was removed and the cultures were washed x4 with ice cold Krebs solution. The cells were removed from the Petri dish by treatment with 1.0mls. of 0.25% trypsin (v/v in $\text{Mg}^{2+}/\text{Ca}^{2+}$ -free Earles Balanced Salt Solution) for 10 min. at 37°C . The trypsin was neutralised by the addition of 2.0 mls. of normal Krebs solution and the cell clumps were dispersed by passage through a wide bore stainless steel needle. A 1.0 ml. portion of the cell suspension was diluted in 9.0 mls. of Isoton counting fluid and used for the estimation of cell number and volume. A further 1.0 mls. of the suspension were mixed with 10.0 mls. of scintillation fluid for radioisotope determination in a packard Tricarb Liquid Scintillation Spectrophotometer (model 3320). The vials were cooled for at least 1hr. in the machine to allow chemical luminescence to subside and the counts obtained were corrected for quench by the external standard ratios method using a quench correction curve

obtained by counting a tritiated sample in the presence of increasing concentrations of serum protein. The ouabain binding was expressed in terms of molecules bound per cell.

Concanavalin A binding

Concanavalin A (Con A) binding was estimated by the method of Noonan and Burger (1973) with the modification that nonspecific Con A binding was measured in the presence of 3mM α -methyl mannose. Unused plastic Petri dishes bind considerable quantities of Con A but this binding is non-specific and is reduced to 2% in the presence of α -methyl mannose. This nonspecific binding is reduced to around 10% when the Petri dishes are used to support the growth of cells. The experimental procedure was as follows: Cultures were washed x4 with Krebs solution and incubated for 10 min. at 0°C in a solution of Krebs containing 200 μ g/ml Con A and ^3H Con A at a specific activity of 0.1 μ Ci/ml. with or without 3mM α -methyl mannose. Preliminary experiments had shown that binding was maximal under these conditions. Preparation of cultures for cell number and radioisotope counting and the measurements themselves were performed as described above for ouabain binding. Quench correction was carried out and the results were expressed in terms of μ g Con A bound/ 10^6 cells. It would have been preferable to express the results in terms of molecules bound/ 10^6 cells but this was not possible due to the heterogeneous molecular weight of the Con A used.

RESULTS

The enzymes investigated in this section may, for convenience, be considered as either intracellular or associated with the cell surface. The activities of (Na+K)-ATPase and 5'-nucleotidase may be considered as cell surface, while acid phosphatase, monoamine oxidase and rotenone insensitive NADH-ferricyanide reductase activities may be considered as intracellular. In view of the reservations expressed earlier, these assumptions may appear arbitrary. They have, however, been made for several reasons: 1) these are the generally accepted locations of these enzymes; 2) at least a large proportion of their activities can be demonstrated in these sites (see Chapter I) and 3) a good deal of lengthy investigation would be required to establish, with certainty, the actual sites of the respective enzymes.

The problems concerning the use of marker enzymes to investigate the effect of culture density on the cell membrane may be avoided by the use of specific ligands for cell surface structures. In this case the use of ouabain and Concanavalin A (Con A) (see Chapter I) which are thought not to enter the cell, will provide more direct evidence for the effects of culture density on the cell surface.

The results reported here deal with the effects of culture density on 8 parameters of 4 different cell lines. In addition, they are also concerned with the effects of serum type on several of these parameters, in both 3T3 and

SV40 3T3 cells. Details of these experiments make unavoidably complicated reading and some preview of the layout of the data may assist the reader. The first part of these results deals with the cell surface enzymes of the 3T3 cells grown in calf serum. The second part deals with the attempts to determine how increasing population density brings about the alterations in the activities of the enzymes. Since, in 3T3 cell lines, it is not possible to relate the (Na+K)-ATPase activity directly to the number of sodium pump sites, the third part outlines the effects of HeLa cell population density on the plasma membrane enzyme activities and relates these to the specific ouabain binding. In order to relate the cell surface alterations to some structural component of the cell surface, the effect of cell population density on the specific ³H Con A binding was also investigated. The fourth part puts the alterations at the cell surface into perspective with occurrences in the rest of the cell by illustrating the effects of culture density on the intracellular enzymes. The final part deals with the alteration in the effects of density observed when the cells are grown in media containing 10% foetal calf serum instead of 10% newborn calf serum.

Cell surface enzymes

The activities of the cell surface enzymes of Py 3T3 cells were observed to decrease as the culture density increased. The protein content of these cells was also observed to decrease (Fig. 4.4.(a)) and the specific activity of the various enzymes is expressed in terms of both cell number

Fig. 4.1 The effect of cell population density on the (Na+K)-ATPase of Py3T3 cells.

The activity is expressed in terms of nM/mg/Hr (Fig.4.1(a)) or nM/10⁶ cells/Hr(Fig. 4.1(b)).

As the culture density exceeds 5×10^4 cells/cm², the specific activity, in terms of total protein, decreases 3.6 fold, from 1720 ± 210 to 490 ± 40 nM/mg/Hr ($p=0.001$ by linear regression analysis). There is a further gradual decrease as the density increases from 5 to 20×10^4 cells/cm² but this is not statistically significant. Fig. 4.1(b) shows a 4.5 fold decrease in activity over the same density range ($p=0.001$ by linear regression analysis). The difference is due to a decrease in cell protein as the cell population density increases (Fig. 4.4).

Each column is the mean of 4-8 experiments and the vertical bars are the standard errors of the means.

Fig.4.1(a)

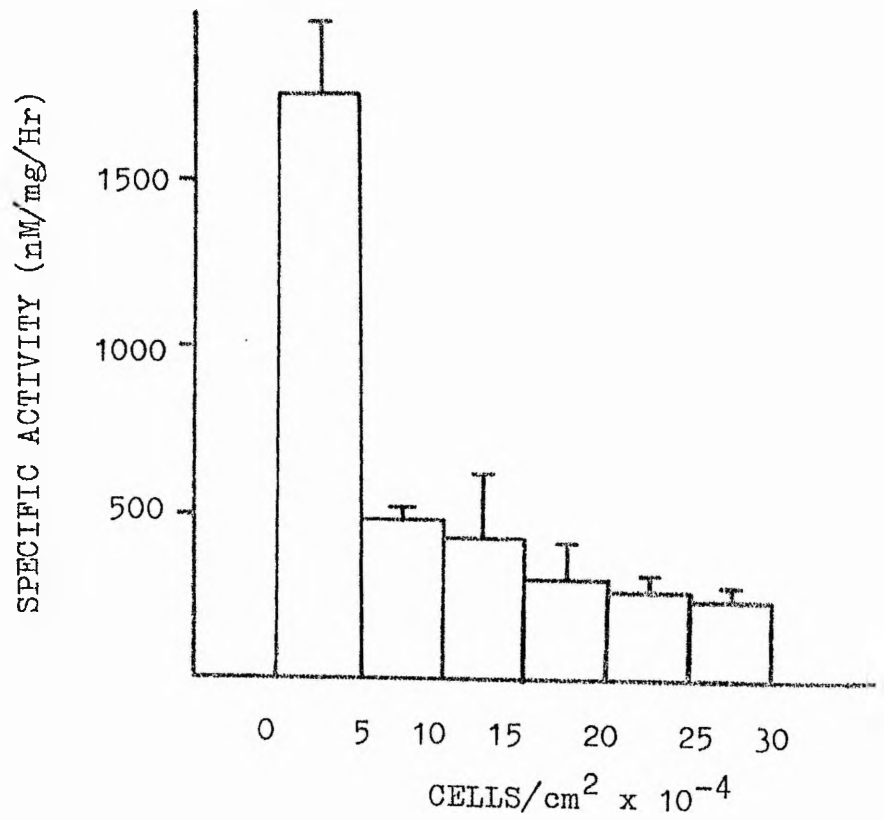


Fig.4.1(b)

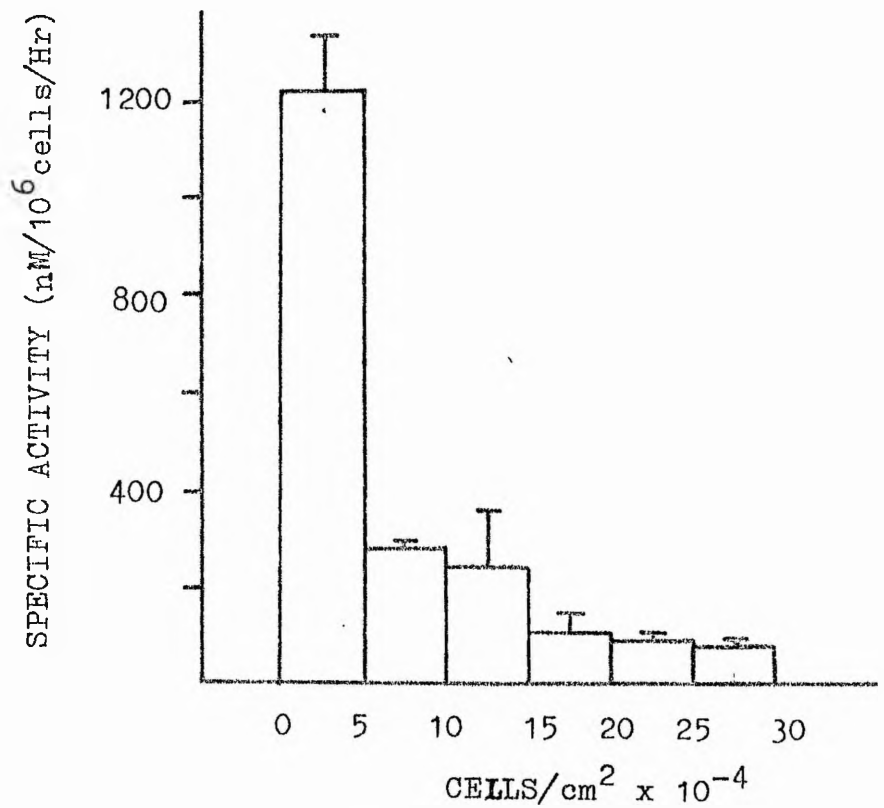


Fig.4.1 The activity of the (Na+K)-ATPase from Py3T3 cells with respect to cell density.

Fig. 4.2 The effect of cell population density on the 5'-nucleotidase activity of Py3T3 cells.

The activity is expressed in terms of nM/mg/Hr(4.2(a)) or nM/10⁶cells/Hr(4.2(b)).

Fig. 4.2(a) shows a gradual reduction in 5'-nucleotidase activity which decreases 2.4 fold as the culture density increases. As the cell population density increases from 0-30 x 10⁴ cells/cm² the 5'-nucleotidase activity decreases from 130[±]30 to 50[±]8 nM/mg/Hr($r=0.02$ by linear regression analysis). In terms of total cell numbers, the activity exhibits a 4.6 fold reduction, from 33[±]27 to 20[±]3 nM/10⁶cells/Hr($r=0.01$ by linear regression analysis. In both cases, the gradual decrease in enzyme activity is in contrast to the sharp reduction observed with the (Na+K)-ATPase activity.

Each column represents the mean of 4-8 observations and the vertical bars represent the standard error of the mean.

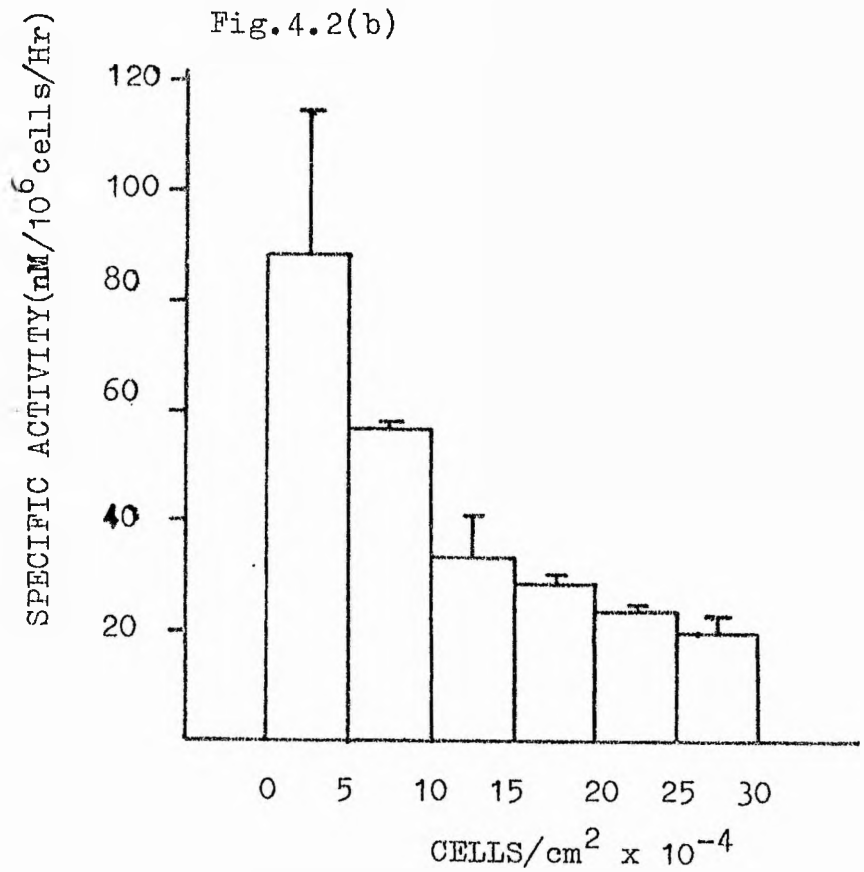
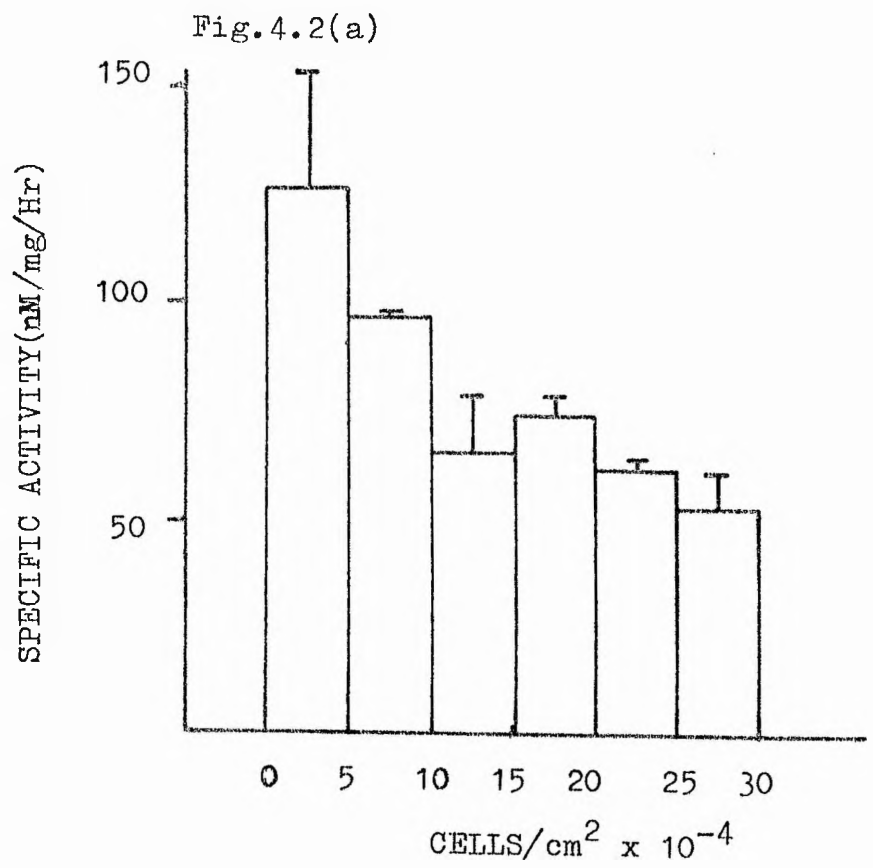


Fig.4.2 The activity of the 5'-Nucleotidase of Py3T3 cells with respect to cell density.

Fig. 4.3 The effect of culture density on the exo-ATPase activity of Py3T3cells.

The activity is expressed in terms of (a) nM/mg/Hr and (b) nM/ 10^6 cells/Hr. In terms of both cell numbers and cell protein there is a significant decrease in enzyme activity as the culture density increases ($p=0.01$ by linear regression analysis). Over the range 0-20 cells/cm², the activity decreases 3.7 fold (531 ± 10 to 143 ± 33 nM/mg/Hr. As the density increases above this level, there is no significant alteration in activity. In terms of cell numbers (b) the pattern of the decrease is similar, but the extent is greater. In terms of cell numbers the activity is reduced 8 fold (376 ± 10 to 46 ± 6 nM/mg/Hr) as the culture density exceeds 5×10^4 cells/cm².

The columns are the mean of 4-8 experiments performed in duplicate and the vertical bars represent the standard error of the mean.

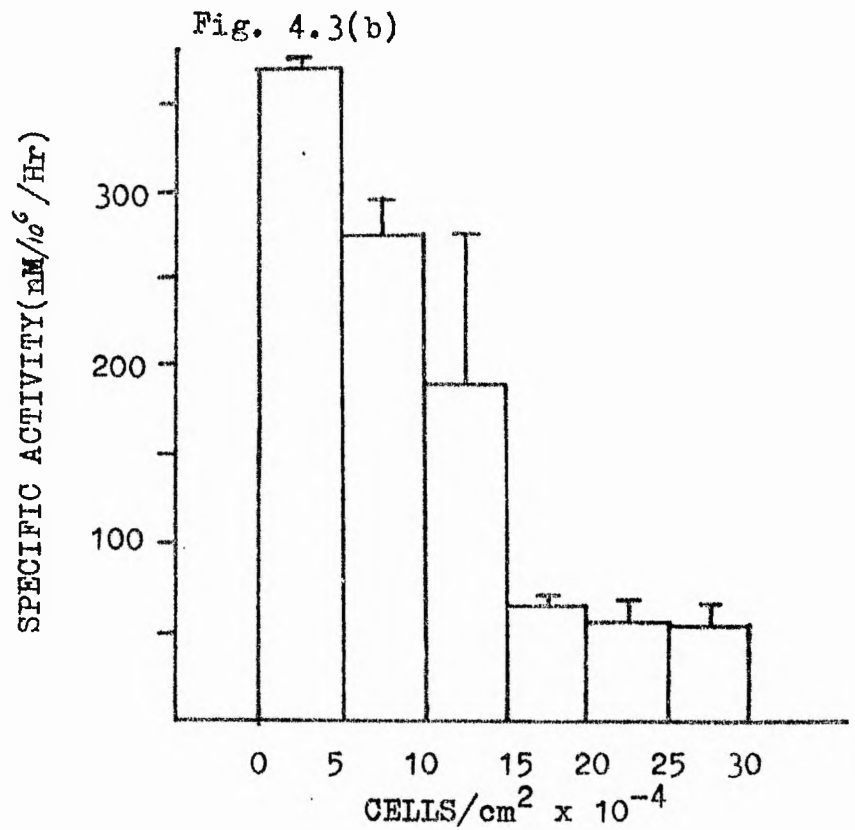
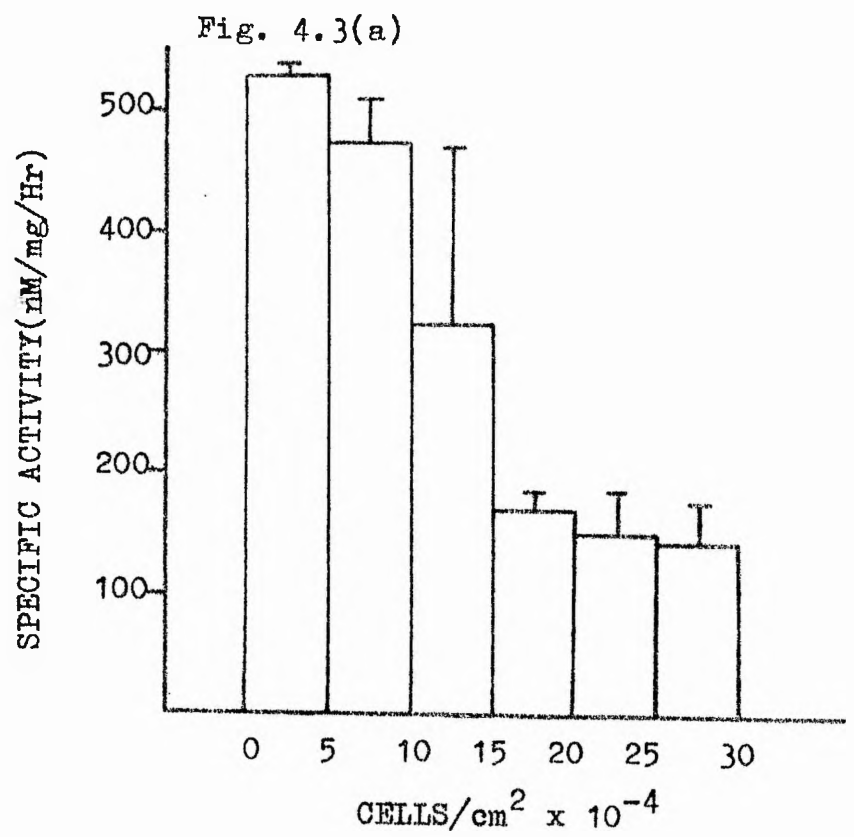


Fig. 4.3 The activity of the exo-ATPase of Py3T3 cells with respect to cell population density.

Fig. 4.4 The effect of cell population density on (a) the cell protein and (b) the cell volume of Py3T3 cells.

As the number of cells per unit surface area increases from 0 to 15×10^4 cells/cm² the cell protein is reduced by 1.8 fold from 0.7 ± 0.2 mg/ 10^6 cells to 0.38 ± 0.09 mg/ 10^6 cells ($p=0.02$ by linear regression analysis). At densities greater than 15×10^4 cells/cm² there is no significant decrease in cell protein.

The cell volume becomes reduced from 1.9 ± 0.07 to 1.5 ± 0.07 mls/ 10^9 cells as the culture density exceeds 25×10^4 cells/cm² ($p=0.02$ by student T test).

The columns are the mean of 4-8 experiments performed in duplicate and the vertical bars represent the standard error of the mean.

Fig.4.4(a)

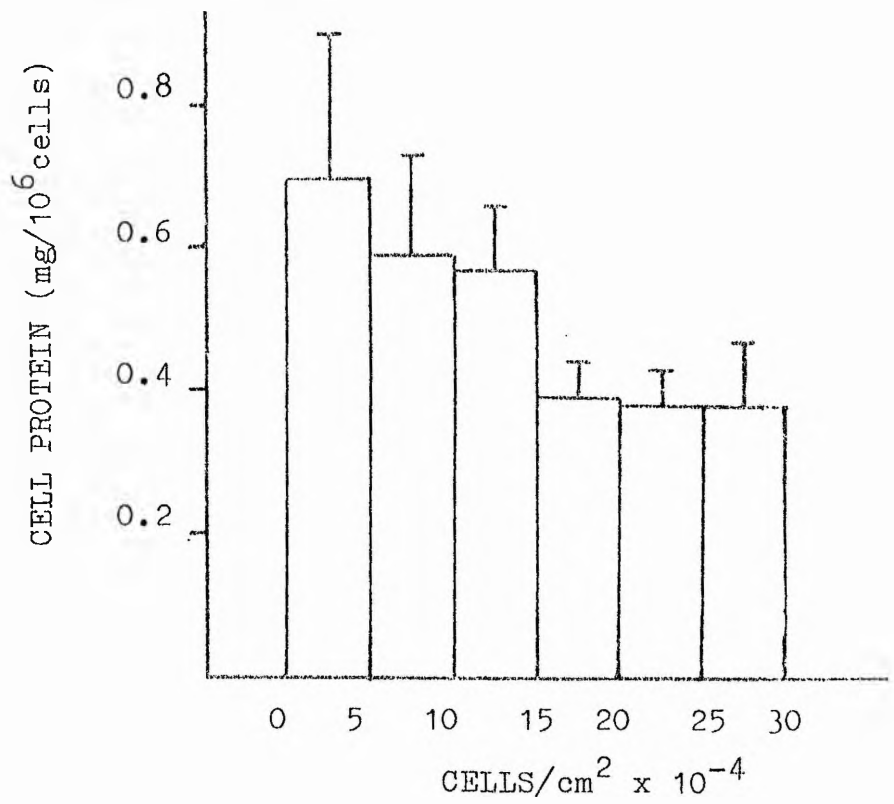


Fig.4.4(b)

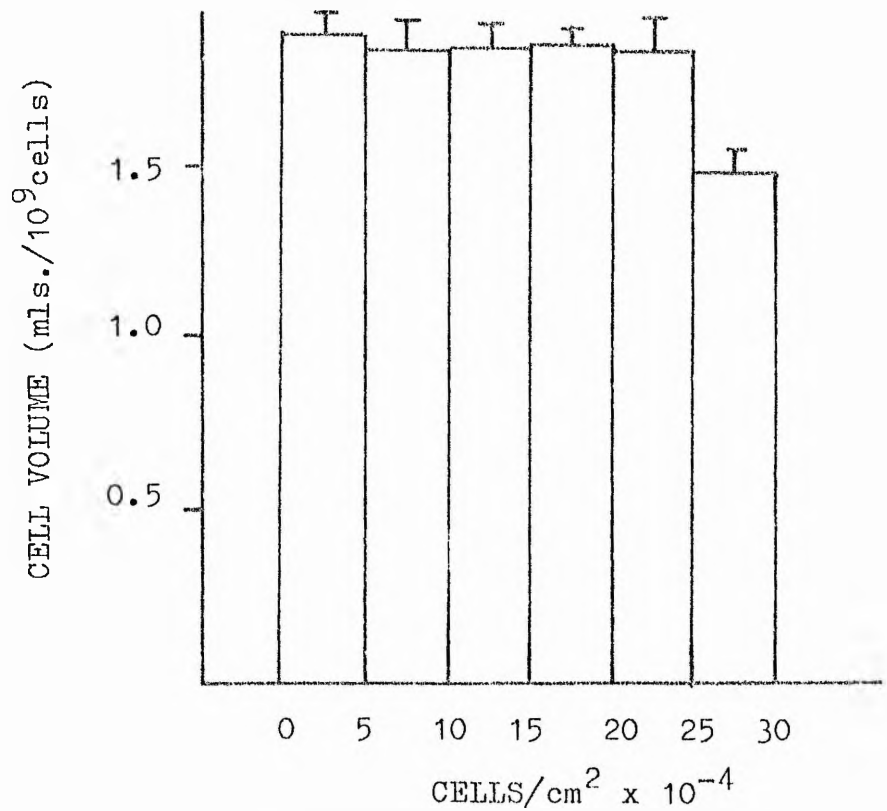


Fig.4.4 The effect of cell density on (a) cell protein and (b) cell volume.

and cell protein. In general the trend is similar but since the cell protein decreases with density, the activity per cell appears to alter to a greater extent. The 3 proposed plasma membrane markers were observed to alter in different ways. Fig. 4.1 shows the effect of culture density on the activity of the (Na+K)-ATPase. There is a large decrease in the activity of the enzyme as the number of cells/cm² exceeds 5×10^4 and, as the cell number increases beyond this level, the activity is not affected. The activity of 5'-nucleotidase is also reduced as the culture density increases (Fig. 4.2); however, in this case the decrease occurs in a gradual fashion. The activity of the 5'-nucleotidase levels off eventually so that the overall reduction in both enzymes is similar. The activity of the exo-ATPase, on the other hand (Fig. 4.3) decreases gradually up to a density of 15×10^4 cells/cm². At this value the activity seems to plateau. Since the hydrolysis of ATP by the exo-ATPase is only a small proportion of the total cell ATPase activity, these measurements will be greatly affected by any cell rupture which may occur. In these experiments, the assay medium was made isotonic in an attempt to prevent cell lysis and Nigrosin staining was performed in order to estimate its extent. The fraction of cells staining positive with Nigrosin proved to be $15 \pm 3\%$ irrespective of the density of the cultures. However, it was difficult to correct for the effects of lysis, since it was impossible to discern whether the lysed cells had died prior to the experiment and had low levels of intracellular ATPases or whether a fraction of the cells which might have an altered enzymic activity was more susceptible to lysis.

If the internal ATPase activity decreased as the density increased then these results would be biased even though the extent of lysis was the same at each density. In any case no attempt was made to correct for the effects of lysis.

Unlike Py3T3 cells, the protein content of SV40 3T3 cells did not alter significantly with culture density (Table 4.2). However, the activities of the cell surface enzymes altered in a manner similar to those of Py3T3 cells. The (Na+K)-ATPase activity (Fig. 4.5) decreased sharply at 5×10^4 cells/cm², while the activity of 5'-nucleotidase decreased gradually with density (Fig. 4.6). The activity of the latter levelled off at high densities, so that the overall alteration in the activities of both enzymes was similar. Due to the difficulties involved in the interpretation of the measurements of the exo-ATPase activity, the effect of SV40 3T3 culture density on this enzyme was not investigated.

During the present investigation, 3T3 cells grew to a final density of 15×10^4 cells/cm² and this value represents the upper limit to density. The effect of culture density on the activities of the cell surface enzymes is shown in Fig. 4.7 and 4.8. In both cases there is an increase in the activity of the enzyme as the culture density exceeded 5×10^4 cells/cm².

The effect of culture density on the activities of the cell surface enzymes in both normal and virus transformed cells is in conflict with that reported in the literature (see Introduction). However, these differences may be resolved by a consideration of the type of serum in which cells have been grown (see later).

Fig.4.5 The effect of culture density on the (Na+K)-ATPase activity of SV40 3T3 cells.

In SV40 3T3 cells there was no significant difference in cell protein as the culture density increased. The results of fig. 4.5 are, therefore, expressed in terms of total protein. As the culture density exceeds 5×10^4 cells/cm² the activity of the (Na+K)-ATPase is reduced by 2.8 fold($p=0.01$ by linear regression analysis) from 492 ± 62 to 169 ± 51 nM/mg/Hr. At densities greater than 5×10^4 cells/cm² there is no significant decrease in the activity of this enzyme.

The columns are the mean of 5-8 experiments performed in duplicate and the vertical bars represent the standard error of the mean.

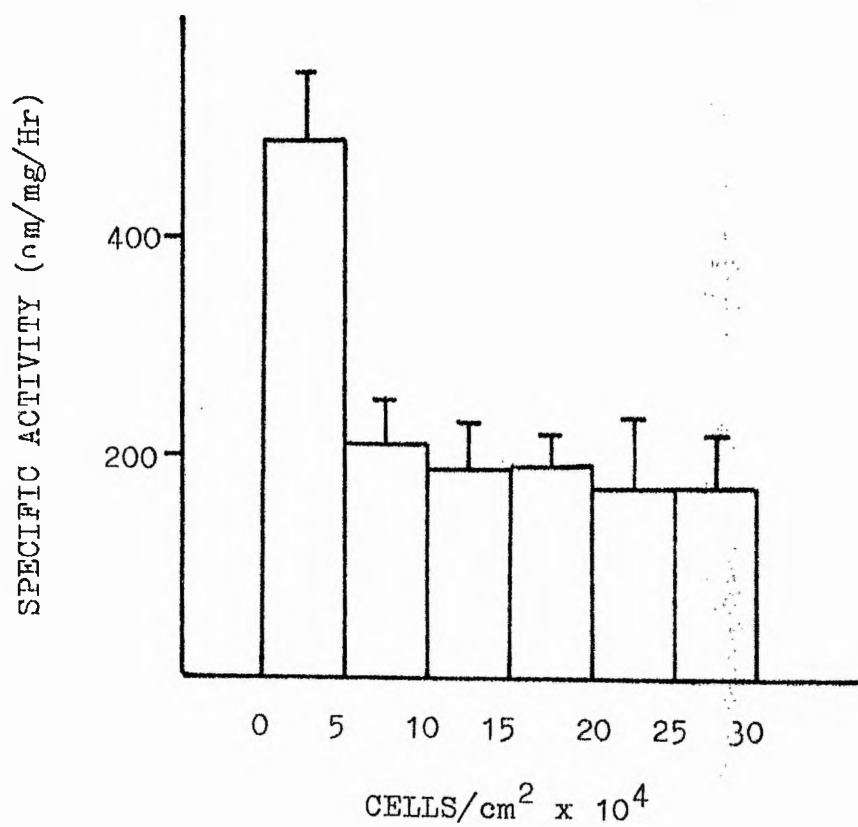


Fig.4.5 The effect of cell density on the (Na+K)-ATPase of SV₄₀T3 cells.

Fig. 4.6 The effect of culture density on the 5'-nucleotidase activity of SV40 3T3 cells.

As the culture density increases from 0-5 to $15-20 \times 10^4$ cells/cm², there is a 4 fold decrease in the 5'-nucleotidase activity, from 480 ± 50 to 108 ± 15 nM/mg/Hr ($p=0.01$ by linear regression analysis). As the cell population density increases above this level, there is no significant alteration in the activity of this enzyme.

The activity is expressed in terms of nM/mg/Hr since the protein content of these cells does not alter significantly with density. The columns are the means of 6-8 experiments performed in duplicate and the vertical bars represent the standard error of the mean.

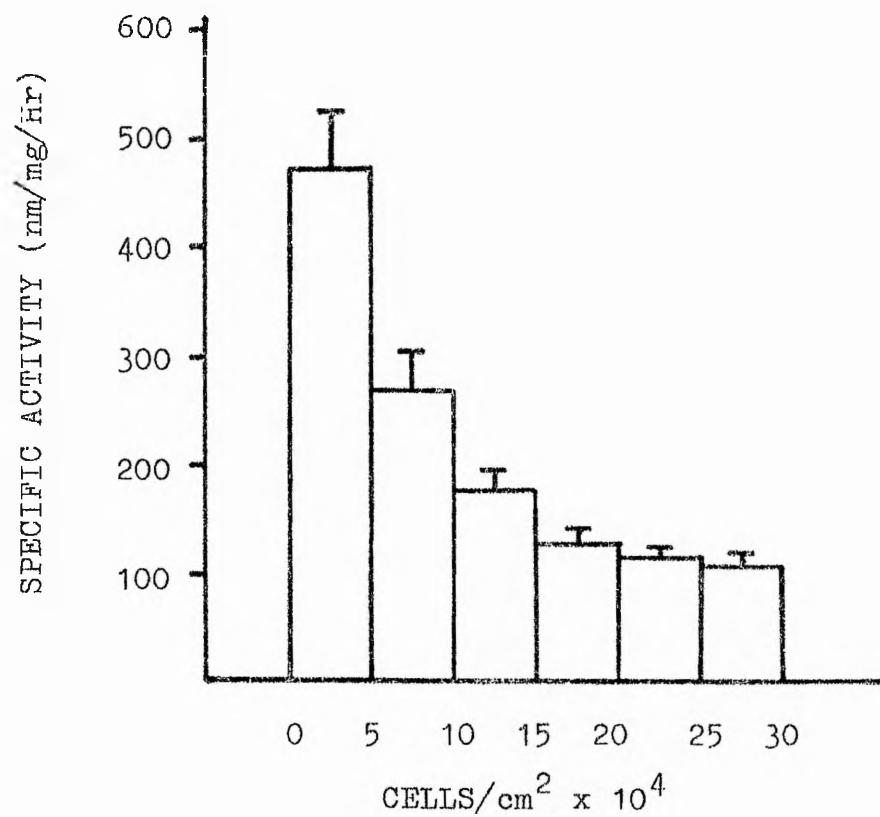


Fig.4.6 The effect of cell density on the 5'-Nucleotidase activity of SV₄₀T3 cells.

Fig.4.7 The effect of culture density on the 5'-nucleotidase activity of 3T3 cells.

As the number of cells/cm² increases above 5×10^4 the 5'-nucleotidase activity increases 3.4 fold, from 200 ± 40 to 730 ± 100 nM/mg/Hr ($p=0.001$ by student's T test). Above 5×10^4 cells/cm², the activity does not alter significantly with density.

The activity is expressed in terms of nM/mg/Hr, since the protein per cell does not alter significantly with density; the columns are the mean of 5-8 experiments and the vertical bars are the standard error of the mean.

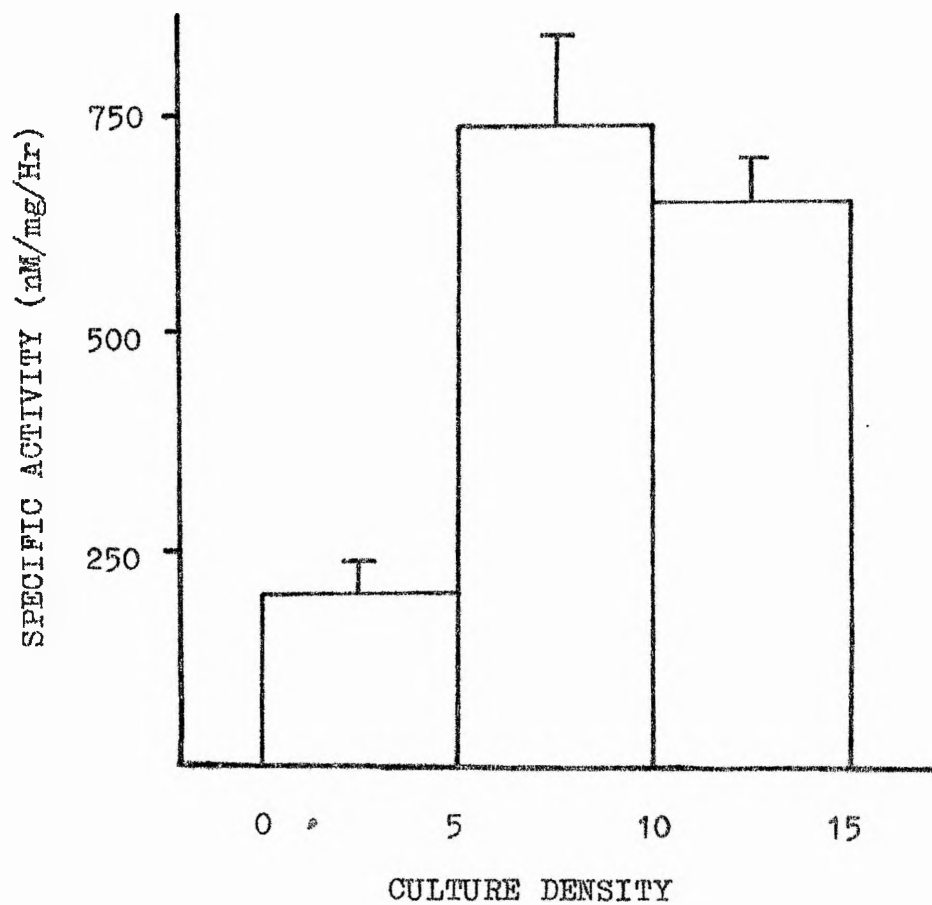


Fig. 4.7 the effect of cell population density on the 5'-nucleotidase activity of 3T3 cells.

Fig. 4.8 The effect of cell population density on the (Na+K)-ATPase activity of 3T3 cells.

As the population density exceeds 5×10^4 cells/cm², the (Na+K)-ATPase activity increases 3.3 fold, from 190 ± 40 to 630 ± 85 nM/mg/Hr ($p=0.001$ by student's T test). As the density increases further, the activity of the enzyme is not significantly affected.

The columns represent the mean of 6-8 experiments and the vertical bars are the standard error of the mean.

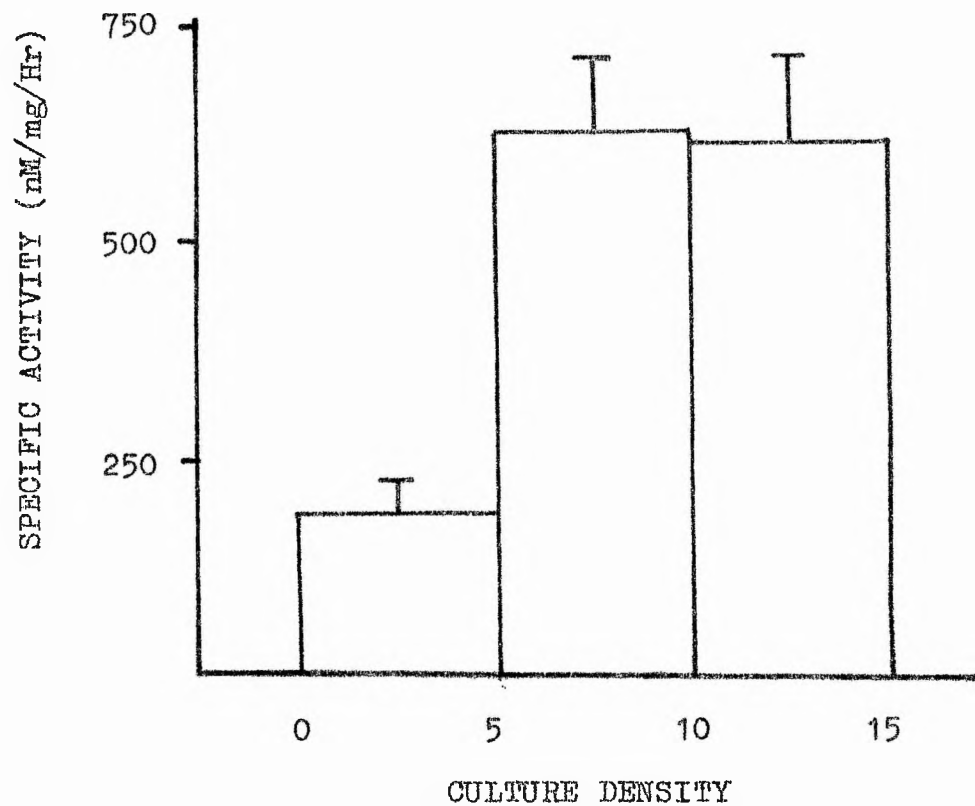


Fig. 4.8 The effect of cell population density on the (Na+K)-ATPase activity of 3T3 cells.

An investigation of the factors likely to cause alterations in the cell surface enzyme activities

The decrease with density, in the activities of the cell surface enzymes of transformed 3T3 cells could be due to an increase in the apparent K_m , a decrease in the V_{max} or the acquisition of a non-linear timecourse. In high density cultures the apparent K_m for the (Na+K)-ATPase is still less than one tenth of the substrate concentration. Even if the apparent K_m had increased dramatically, it would still not be high enough to alter the observed rate of hydrolysis because the measurements would still be made on the *plateau* portion of the Michaelis-Menten curve. The decrease in activity with density cannot, therefore, be attributed to an increase in the apparent K_m . In addition, the rate of reaction is linear at high densities for at least 30 min. so that the reduction in (Na+K)-ATPase activity cannot be attributed to the acquisition of a non-linear timecourse. It appears, therefore, that the decrease in activity of this enzyme is due to a reduction in the V_{max} of the reaction. It is conceivable that the increase in activity observed in 3T3 cells may be due to a decrease in the K_m (app.) and this possibility was not excluded in the present investigation. A sudden reduction in the apparent V_{max} for the reaction might occur without affecting the sodium pumping activity, if the properties of the cell surface altered so that, at high population densities, a portion of the enzymic activity became cryptic during homogenisation. It has been reported that detergent may increase 5'-nucleotidase (Konopka et al 1972)

and (Na+K)-ATPase activity (Stahl 1973). In SV40 3T3 cells the activity of neither of these enzymes is increased in the presence of detergent. Fig. 4.9 shows the effect of 2-deoxycholate on the activities of 5'-nucleotidase and (Na+K)-ATPase in SV40 3T3 cells from high density cultures. As the deoxycholate concentration was increased, the activity of the (Na+K)-ATPase was greatly reduced, while the activity of 5'-nucleotidase was not markedly affected even at detergent concentrations as high as 0.2% (W/V).

Elligsen et al (1974) have reported that the decrease in activity of the (Na+K)-ATPase of 3T3 cells with confluence may be reversed by the addition of serum. However, in the experiments reported here, the addition of serum to, or the omission of serum from the growth medium of SV40 3T3 cells had the same general effect on all of the enzymes measured. This effect is shown in Figs. 4.10 and 4.11. In terms of cell protein (Fig. 4.10) none of the enzymes appears to alter significantly from control values. However, the protein content of the cells was observed to alter with the serum content of the growth medium. Therefore, when the activities are considered in terms of total cell numbers, they appear to increase as the serum content increases and decrease as the serum content decreases (Fig. 4.11). In those cultures treated with 0% serum for 18hr. the activity per cell decreased to around 50% of control values, while those cultures treated with 20% serum possessed activities of most enzymes at levels of 40-80% greater than control values. The effect of serum, therefore, appears indiscriminate, in that it

Fig. 4.9 The effect of detergent on the activity of the 5'-nucleotidase and (Na+K)-ATPase of SV40 3T3 cells from high density cultures.

○ :- 5'-nucleotidase activity; nM/mg/Hr.

● :- (Na+K)-ATPase activity; nM/mg/Hr.

Deoxycholate at concentrations of upto 0.2%(w/v) has no effect on the 5'-nucleotidase activity. However, the (Na+K)-ATPase activity is greatly reduced by deoxycholate at low concentrations. There is no increase in activity of either enzyme over the concentration range shown.

The points are the mean of 3 experiments performed in duplicate and the vertical bars are the standard error of the mean.

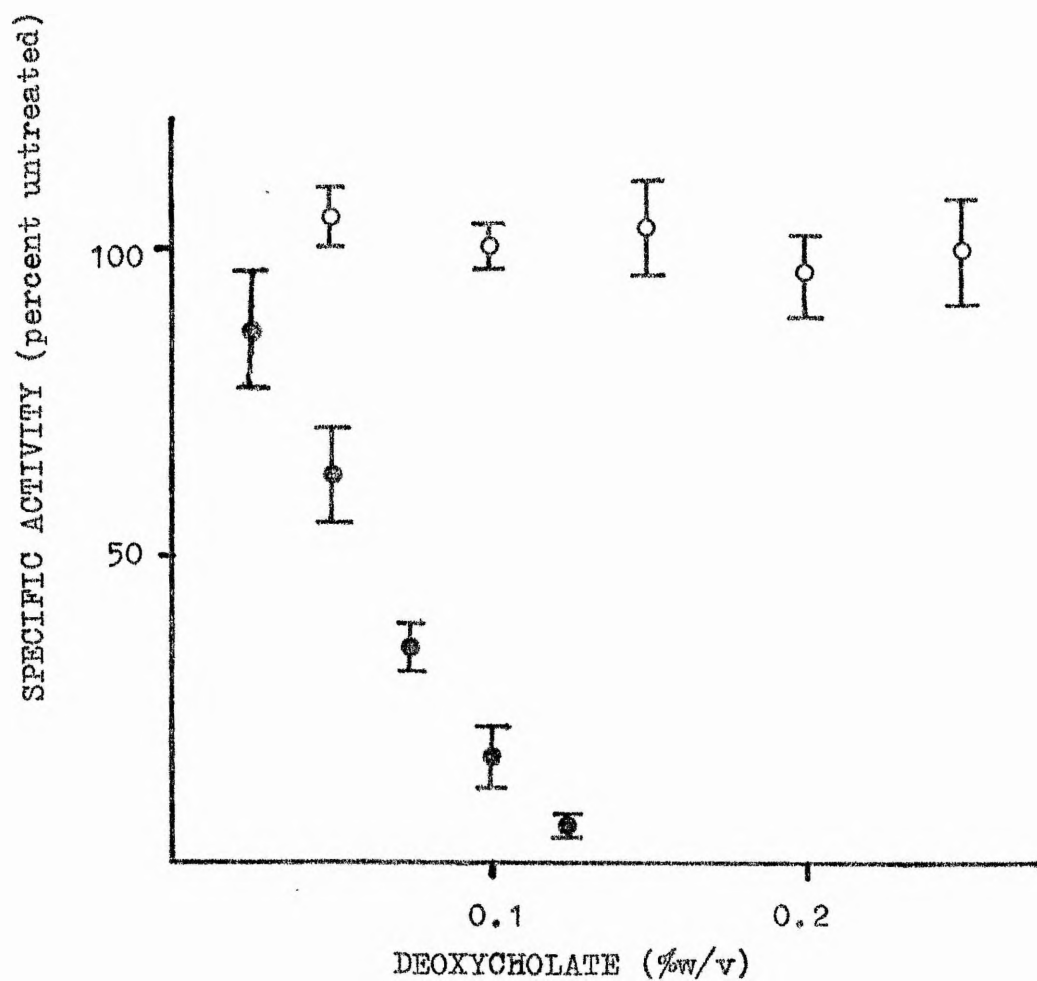


Fig. 4.9 The effect of detergent on the activity of the (Na+K)-ATPase and 5'-nucleotidase of SV40 3T3 cells from high density cultures.

Fig. 4.10 The effect of serum on various properties of SV40 3T3 cells.

In terms of cell protein, the only significant effect of serum is an increase in the activity of the (Na+K)-ATPase and an increase in cell protein itself ($p=0.03$ and 0.001 respectively). The other measurements do not alter significantly from control values.

The enzyme activities were calculated as nM or $\mu\text{M}/\text{mg}/\text{Hr}$ and expressed as percentages of untreated control values. The columns are the mean of 3 experiments and the vertical bars are the standard errors of the means.

A:- 0% serum

B:- 10% serum

C:- 20% serum

Vol:- cell volume in $\text{mls.}/10^9$ cells

(Na+K):- (Na+K)-ATPase activity

5'-NT:- 5'-nucleotidase activity

NADH:- rotenone insensitive NADH-ferricyanide reductase

MAO:- monoamine oxidase activity

AP:- acid phosphatase activity

Prot:- cell protein in $\text{mg}/10^6$ cells

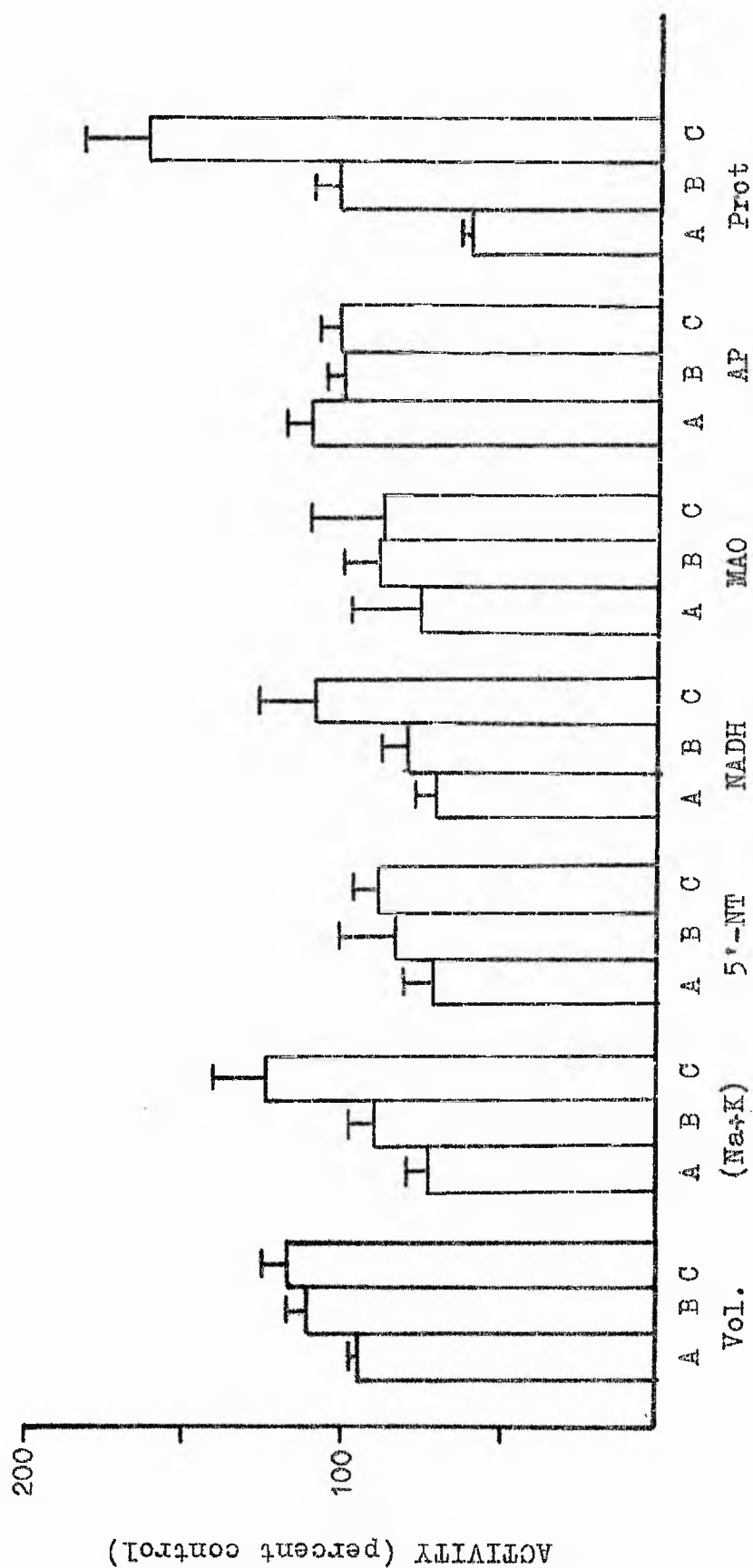


Fig. 4.10 The effect of serum concentration on various properties of SV40 3T3 cells

Fig. 4.11 The effect of serum on various properties of SV40 3T3 cells.

In terms of cell numbers, the activity of all of the enzymes measured increases as the serum increases and decreases as the serum concentration decreases ($p < 0.05$ in all cases). When the cells are incubated with 0% serum, the activities of most of the enzymes decrease to around 40% of control values, and when the serum concentration is increased to 20%(v/v) the activities increase, from 40% in the case of 5'-nucleotidase and monoamine oxidase to 100% in the case of the (Na+K)-ATPase. In most cases, the replacement of the normal growth medium by fresh medium containing 10% serum brings about a slight decrease in activity, but this effect is not statistically significant.

The figure is labeled as Fig. 4.10; the columns are the mean of 3 experiments and the vertical bars are the standard error of the mean. The enzyme activities were calculated as nM or $\mu\text{M}/10^6$ cells/Hr and expressed as percentages of control values.

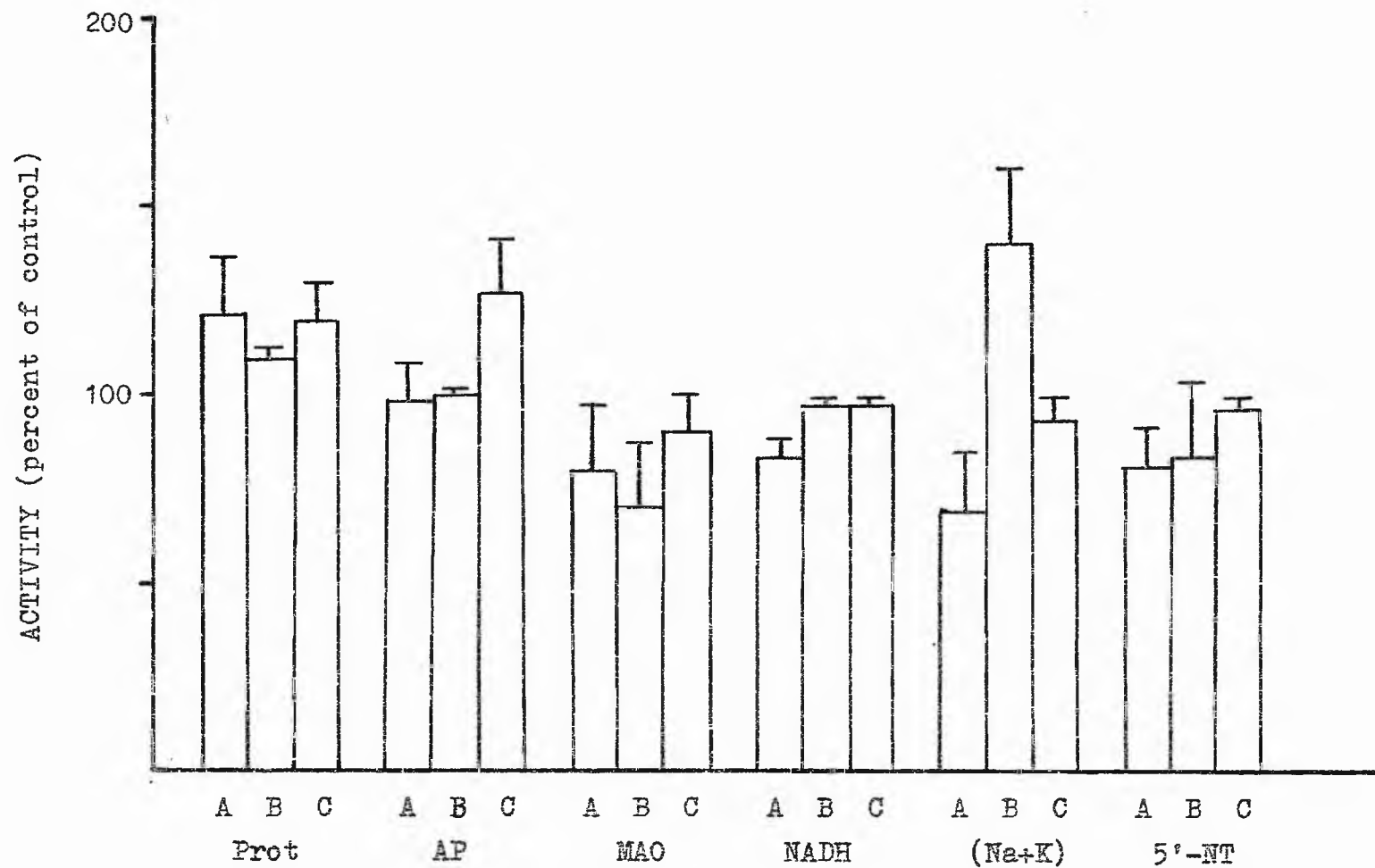


Fig. 4.12 The effect of trypsinization and medium replacement on various properties of SV40 3T3 cells.

brings about an increase in the activity of all of the enzymes investigated.

The alterations in enzyme activities which occur when cells are subcultured might be associated with either the addition of fresh medium or with the disruption of cell-cell contacts. Since fresh medium would not reverse the effects of population density then it might be expected that trypsinisation would. Fig. 4.12 shows that trypsinisation per se did not reverse the effects of increasing culture density. When cultures were trypsinised and treated with fresh growth medium, the protein per cell and the (Na+K)-ATPase activity increased. Though monoamine oxidase activity was observed to decrease under these conditions, the new level was not significantly different from that obtained when trypsin was added to otherwise undisturbed cultures. When cultures were treated with conditioned growth medium, the protein per cell increased while the activities of the (Na+K)-ATPase, 5'-nucleotidase and NADH-ferricyanide reductase decreased. The addition of trypsin to control cultures resulted in an increase in cell protein and acid phosphatase activity. It would appear that the only consistent response to trypsin is an increase in cell protein. If cell contacts were responsible for the alterations associated with density, then disruption of these contacts would be expected to reverse the effects of density. The only indication that this might be the case, is the specific increase in (Na+K)-ATPase activity seen during treatment of trypsinised cultures with fresh medium. This observation suggests that both disruption of cell-cell contacts and the addition of fresh

Fig. 4.12 The effect of trypsinization and medium replacement on various properties of SV40 3T3 cells.

When cultures are trypsinized and treated with fresh growth medium containing 10% serum, the activity of the (Na+K)-ATPase increases to 140% of untreated controls. When trypsin is added to otherwise untreated cultures, the acid phosphatase activity increases to 130% ($p < 0.05$ in both cases).

The values are expressed as nM or $\mu\text{M}/\text{mg}/\text{Hr}$ as a percentage of untreated controls. The nomenclature is the same as Fig. 4.10 except for:-

- A:- trypsinized cultures to which the conditioned growth medium has been readded.
- B:- trypsinized cultures treated with fresh growth medium containing 10% calf serum
- C:- undisturbed cultures to which trypsin has been added.

The columns are the mean of 3 experiments and the vertical bars are the standard error of the mean.

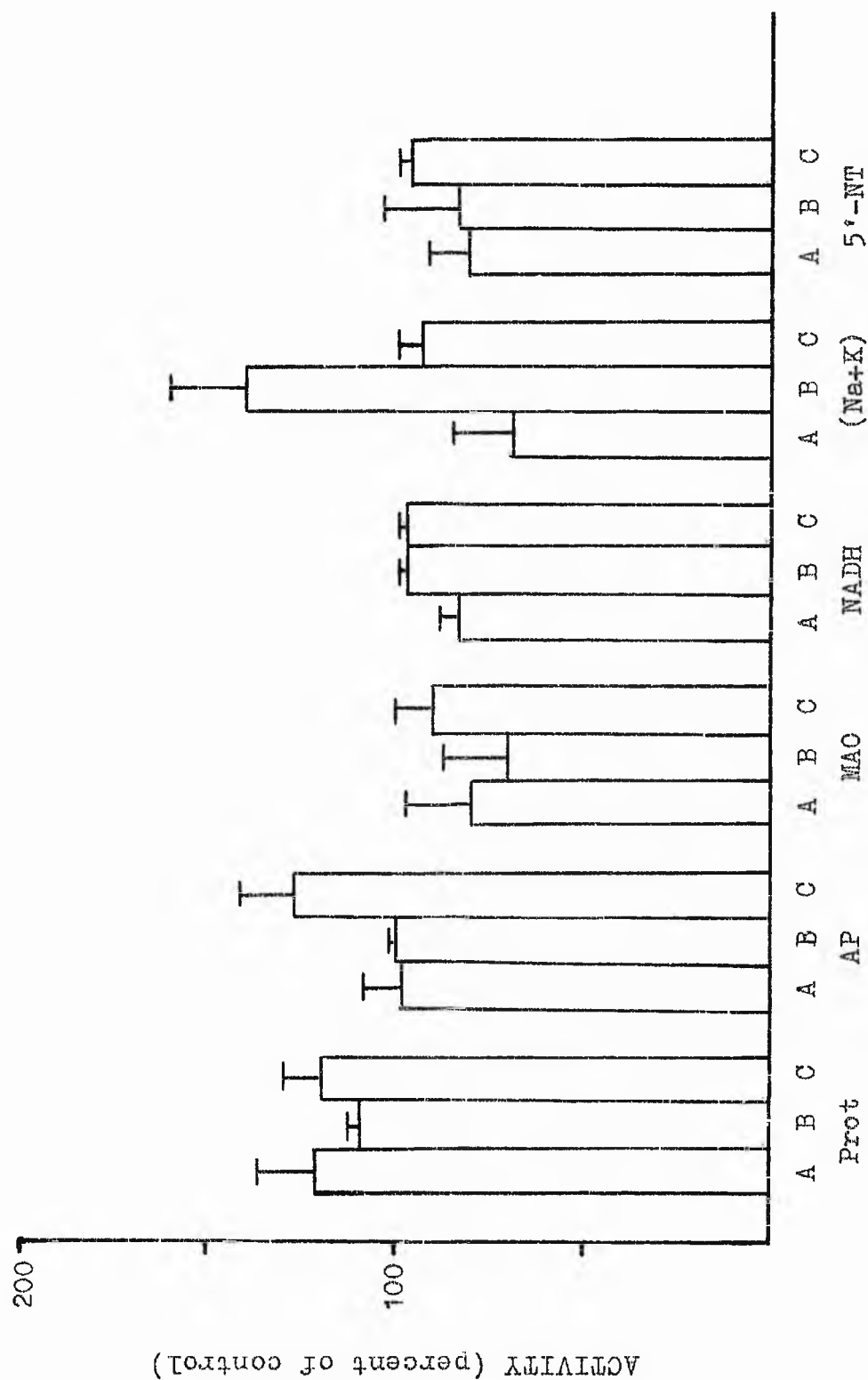


Fig. 4.12 The effect of trypsinization and medium replacement on various properties of SV40 3T3 cells.

medium might be required to reverse some of the effects of cell population density.

Two possible conclusions may be drawn from the above results: 1) that the trypsinisation conditions used did not effectively disrupt the cell-cell contacts which bring about the density associated alterations. The trypsinisation conditions used here are the same as those routinely used for subculturing, but in these experiments trypsinisation was not followed by 'blasting' (see Methods section Chapter I), and it is conceivable that it is the process of 'blasting' which is required for these alterations to occur; 2) that the timecourse of these experiments did not permit the observation of the full alterations. The reversal of the effects of density which occurs when the cells are subcultured, may require several days to become manifest (Horvat and Acs 1974). If this were the case, the 18hr. incubation in these experiments would not be sufficiently long to allow the new steady state to become established. However, it was thought that if the cells were incubated for longer periods, the cell-cell contacts might have become reestablished and the effects of trypsinisation might not have been observed.

The cell surface enzymes of HeLa cells

Although there are density associated alterations in the activities of the (Na+K)-ATPase of transformed 3T3 cells grown in calf serum, the effect of culture density on the active $^{86}\text{Rb}^+$ transport was not investigated here; these experiments were already being undertaken in this laboratory. It would have been instructive, therefore, to estimate the actual

number of sodium pump sites on the surface of the 3T3 cells, but this is not possible due to their very low sensitivity to ouabain. In HeLa cells the method of estimating the number of pump sites is well established and routinely used in this laboratory. Therefore, it was decided to use HeLa cells to establish the relationship between the (Na+K)-ATPase activity and the number of sodium pump sites. Fig. 4.13 shows the alteration in the activities of 5'-nucleotidase and (Na+K)-ATPase with HeLa cell culture density. The alterations follow the same pattern as in the transformed 3T3 cell lines, there is an initial decrease in the level of (Na+K)-ATPase as the culture density exceeds $5 \times 10^4 \text{ cells/cm}^2$, while the activity of 5'-nucleotidase follows a more gradual decline. In cells from both low and high density cultures, the rate of 5'-nucleotidase reaction is linear for at least 30 min. (Fig. 4.14) but the rate of reaction is reduced as the density exceeds $5 \times 10^4 \text{ cells/cm}^2$. When the kinetics of the enzyme in low and high density cultures are compared (Fig. 4.15) it is apparent that the decrease is due to a three fold reduction in the V_{max} of the reaction. Although the apparent K_m increases with density, the alteration is too small to affect the rate of reaction appreciably at substrate concentrations of 5mM.

While Glick (1976) has observed the activity of the (Na+K)-ATPase in all cell fractions, the enzyme should be predominately associated with the cell surface and with the sodium pumps in particular. As previously stated, it was not possible to measure the latter in mouse cells. However, in HeLa cells the specific ouabain binding decreased

Fig. 4.13 The effect of culture density on the 5'-nucleotidase and (Na+K)-ATPase activities of HeLa cells.

Fig. 4.13(b) shows the effect of cell population density on the activity of 5'-nucleotidase. The activity, in terms of nM/mg/Hr, decreases gradually as the culture density increases. The overall reduction is 2 fold, from 800 ± 60 to 360 ± 30 nM/mg/Hr, as the density increases from 0 to 30×10^4 cells/cm².

Fig. 4.13(a) shows the effect of density on the activity of the (Na+K)-ATPase. In this case, the activity decreases 3.5 fold as the culture density exceeds 5×10^4 cells/cm² (from 1200 ± 110 to 340 ± 40 nM/mg/Hr).

In both cases $p=0.001$ by linear regression analysis. The columns are the mean of 5-8 experiments performed in duplicate and the vertical bars are the standard errors of the mean.

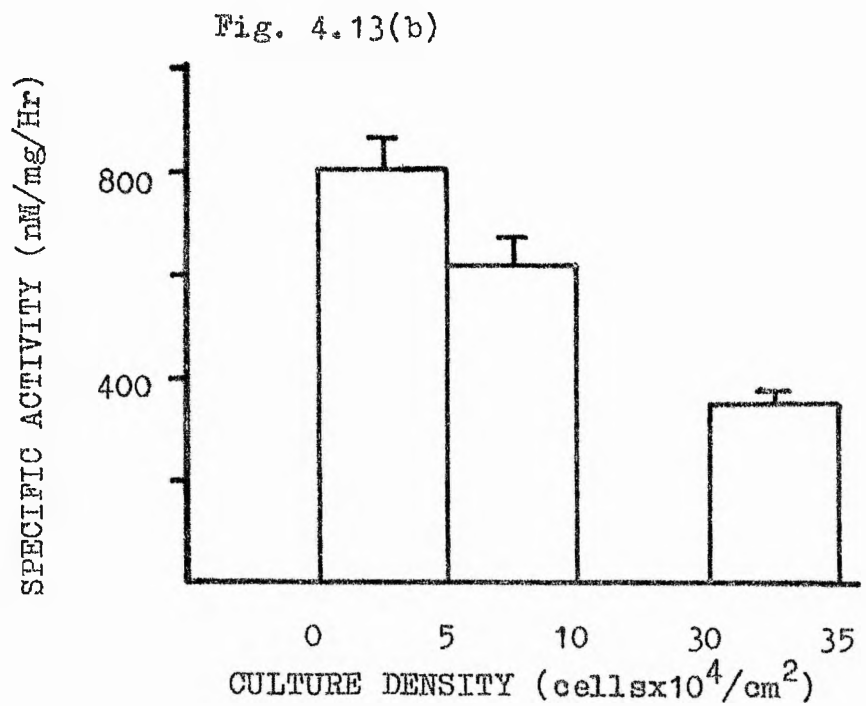
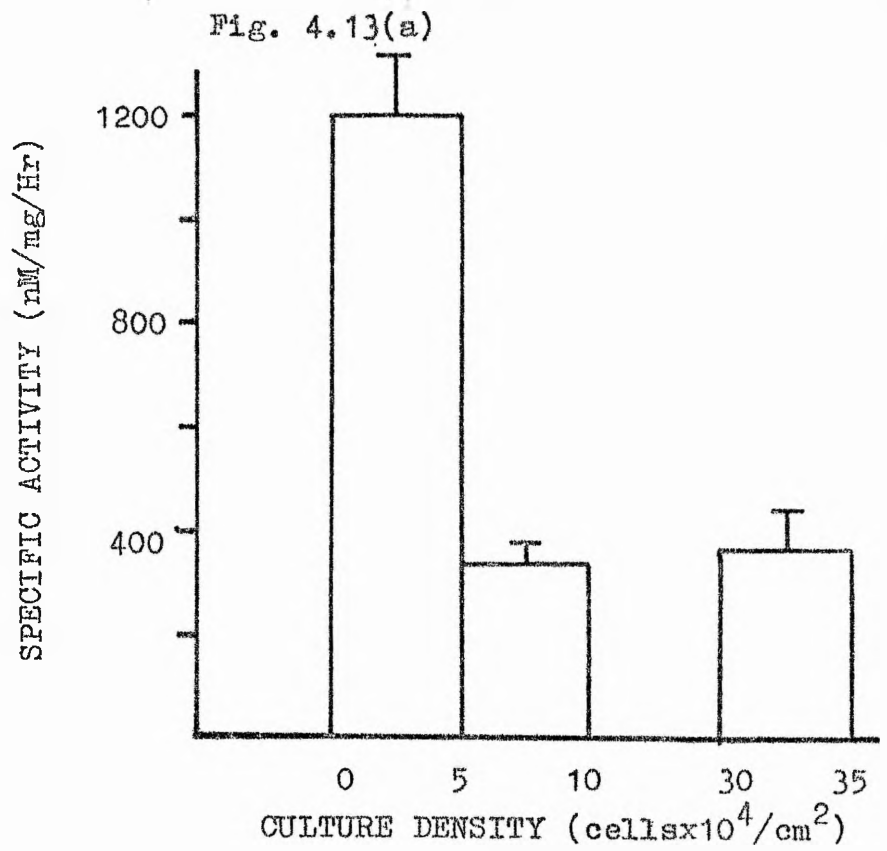


Fig. 4.13 The effect of density on the (Na+K)-ATPase and 5'-nucleotidase of HeLa cells.

Fig. 4.14 The timecourse of hydrolysis of 5'AMP by HeLa cells from low and high density cultures.

- :- low density cultures($< 5 \times 10^4$ cells/cm²)
- :- high density cultures($> 25 \times 10^4$ cells/cm²)

The timecourse of hydrolysis of 5'AMP by high and low density cultures is linear for at least 30min. However, the rate of hydrolysis by low density cultures is 1.8 times faster than by high density cultures($p=0.02$ by regression analysis).

The points are the mean of 3 experiments and the vertical bars are the standard error of the mean.

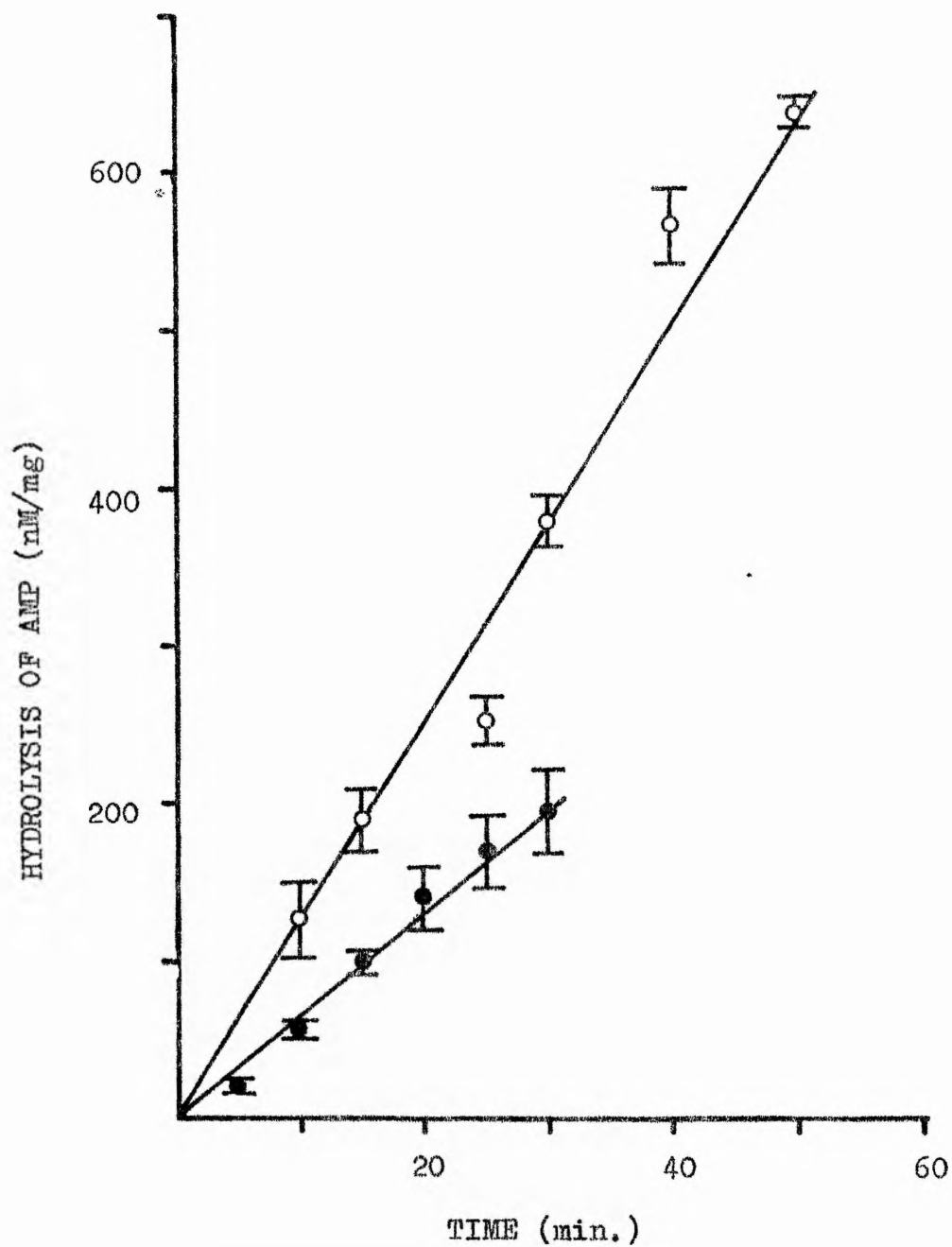


Fig. 4.14 The timecourse of hydrolysis of 5'AMP by HeLa cells from low and high density cultures.

Fig. 4.15 Kinetics of the 5'-nucleotidase of HeLa cells with respect to cell population density.

△ :- low density cultures ($< 5 \times 10^4$ cells/cm²)

▲ :- high density cultures ($> 5 \times 10^4$ cells/cm²)

The rate of hydrolysis of AMP decreases with increasing culture density (Fig. 4.14). The reduction in rate is associated with a decrease in the apparent K_m and an increase in V_{max} for the reaction. The former increases from 0.17 ± 0.06 to 0.47 ± 0.04 mM and the latter decreases from 1050 ± 40 to 350 ± 50 nM/mg/Hr. In the presence of 5mM AMP, the substrate concentration routinely used, the increase in K_m will not be sufficient to cause a marked reduction in the rate of reaction and the decrease in activity must therefore be due to the decrease in V_{max} .

The points are the mean of 3 experiments and the vertical bars are the standard errors of the means. The slopes and intercepts were calculated by linear regression analysis and the lines were drawn by eye.

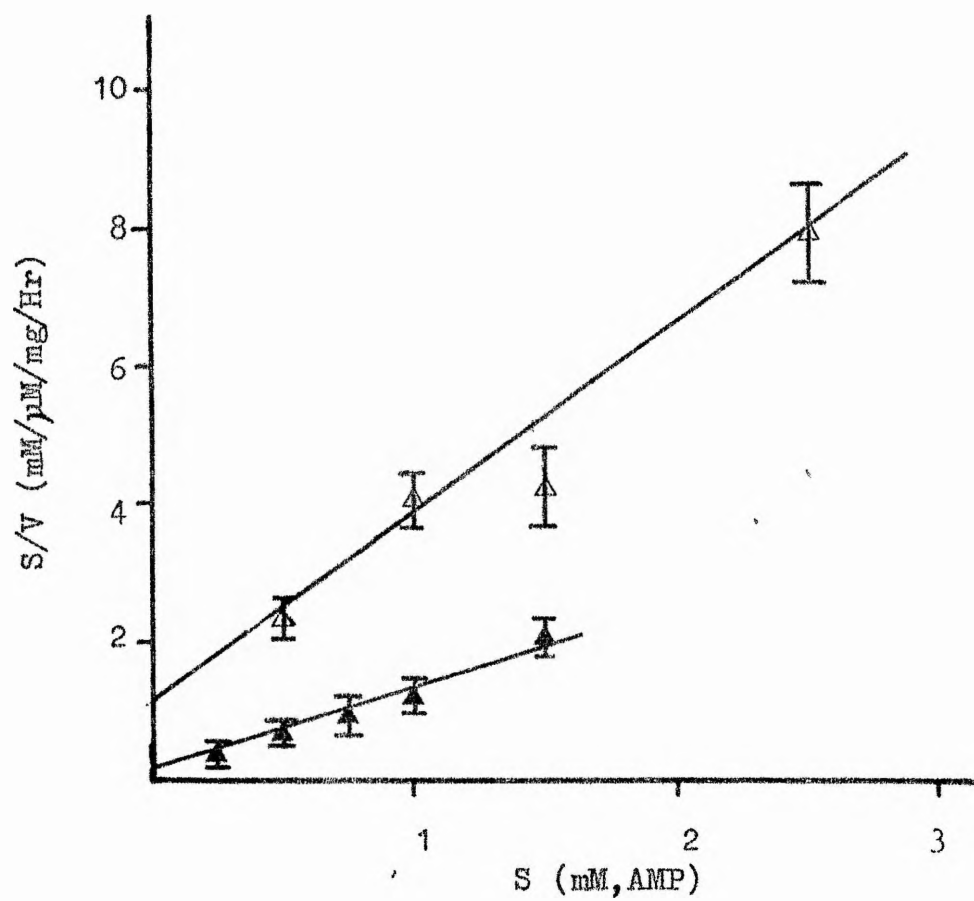


Fig. 4.15 Kinetics of 5'-nucleotidase of HeLa cells from low and high density cultures.

by 2 fold as the culture density increased from $2-7 \times 10^4$ cells/cm² (Fig. 4.16). It would appear that the effects of culture density on the (Na+K)-ATPase activity and specific ouabain binding do not exactly correspond, either in extent or the density of onset. This relationship need not be linear. Cook (1977) has suggested that sodium pumps at the cell surface are turned over, i.e. withdrawn into the cell, with a half time of several hours, to be replaced by sodium pumps, presumably from an internal pool. Though the mechanism which he suggests is in dispute (Lamb, personal communication) an intracellular pool of membrane precursors may exist. If this is indeed the case, then alterations in this pool could occur, without altering the specific ouabain binding, and alterations to the specific ouabain binding need not be associated with changes in the total cell (Na+K)-ATPase.

Concanavalin A binding

It would have been instructive, during these experiments, to measure the cell surface area in relation to population density and compare the values to the other properties investigated. However, in addition to being beyond the scope of the present investigation these experiments were already being performed in this laboratory. Instead, an attempt was made to measure the effect of population density on some known component of the cell surface. Measurements of specific Concanavalin A (Con A) binding promised to prove fruitful in this respect since tritiated Con A of high specific activity was commercially available and a simple experimental

Fig. 4.16 The specific ouabain binding to HeLa cells with respect to cell population density.

As the culture density increases from 1 to 7×10^4 cells/cm² the specific ouabain binding gradually decreases 2 fold (from 204 ± 24 to $102 \pm 10 \times 10^6$ molecules/cell; $p=0.01$ by student's T test).

The columns are the mean of 4 experiments and the vertical bars are the standard errors of the means.

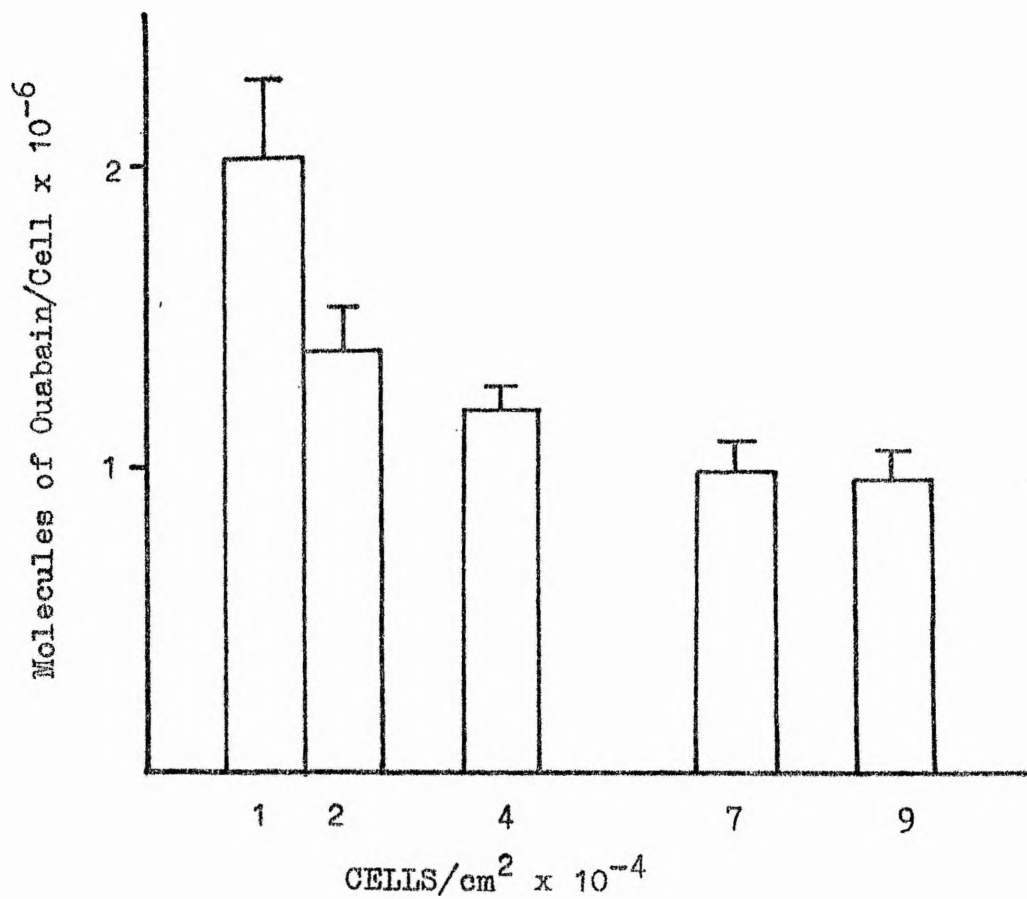


Fig. 4.16 Specific ouabain binding to HeLa cells at different population densities

procedure had already been established (Noonan and Burger 1973). Con A is an agglutinin isolated from Jack Bean meal which acts as a ligand for α -methyl mannose residues at the cell surface. The specific hapten for the agglutinin methyl-D-glucopyranoside (α -MM), inhibits specific Con A binding and the difference between the uptake of Con A in the presence and absence of α -MM is a measure of the specific Con A binding. At 0°C the agglutinin will be unable to penetrate the cell membrane and the specific binding of Con A to cultured cells will therefore reflect the number of available mannose residues at the cell surface.

Fig. 4.17 shows the total and non specific Con A binding to HeLa cells at different culture densities. The specific binding decreases by some 25% over the density range investigated. This reduction represents a decrease in the number of accessible mannose residues at the cell surface, which may occur either by removal of plasma membrane into the cell or by chemical modification of the cell surface. If the alteration in Con A binding were linearly related to a decrease in the cell surface area, the decrease could not account for the reduction in specific ouabain binding or (Na+K)-ATPase activity. However, the 5'-nucleotidase activity is reduced by the same extent as Con A binding over the same density range and these measurements may directly reflect alterations in the cell surface area. If this is the case then the decrease in the number of ouabain binding sites must be attributed to a specific effect of density rather than being related to an overall reduction in cell surface area or function.

Fig. 4.17 Concanavalin A binding to HeLa cells
with respect to cell population density.

Both the total and non-specific Con A binding decrease as the culture density increases ($p=0.02$ by linear regression analysis). The total binding decreases by 24% (from 11.5 ± 1.0 to 8.8 ± 0.7) as the density increases from 2 to 6×10^4 cells/cm² and the non-specific binding decreases by 60% over the same range (from 1.6 ± 0.14 to 0.6 ± 0.1 μ g/ 10^6 cells).

The columns are the mean of 4 experiments and the vertical bars are the standard errors of the means. The cross-hatched columns are the non-specific binding values.

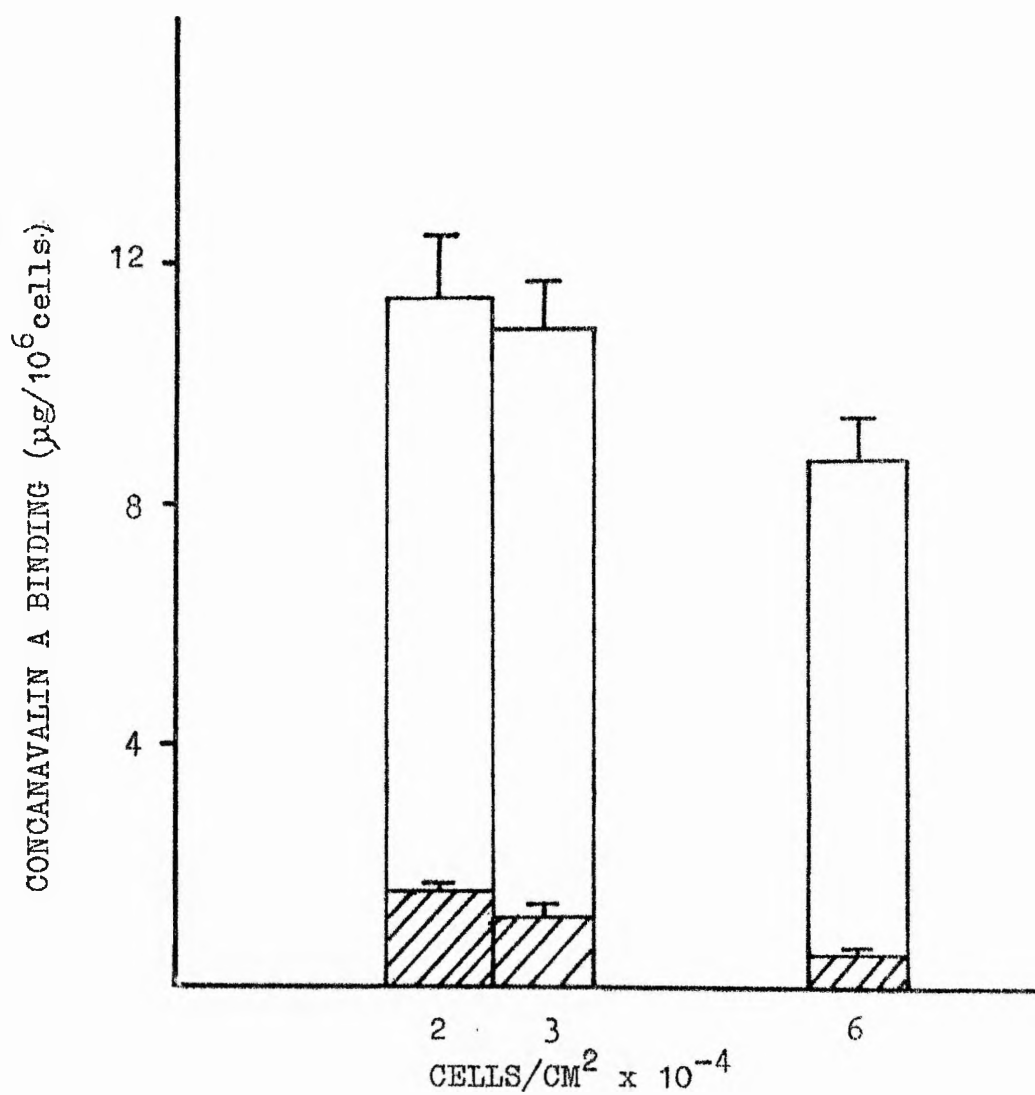


Fig. 4.17 The effect of cell population density on Concanavalin A binding to HeLa cells.

The alterations in Con A binding with density are not restricted to HeLa cells. The phenomenon has a counterpart in normal 3T3 cells and in SV40 3T3 cells. In SV40 3T3 cells (Fig. 4.18) there is a 50% reduction as the density increases from $4-16 \times 10^4$ cells/cm² but, as the cell density exceeds 5×10^4 cells/cm², the specific Con A binding is reduced by only 35%. This decrease is not sufficient to account for the alterations in enzyme activities which occur at this density.

The quantity of specific Con A binding to 3T3 cells is also affected by culture density (Fig. 4.19). In this case there is a 50-60% decrease as the culture density increases from $2-7 \times 10^4$ cells/cm². In these cells, the activities of the cell surface ^{ENZYMES} increase with density and cannot be related to a decrease in Con A binding.

Cell volume and protein

The reductions in Con A binding reported above are suggestive of decreases in the cell surface areas. However, measurements of cell volume did not support this suggestion. In Py3T3 cells (Fig. 4.4(b)) the volume did not alter significantly with culture density until the number of cells exceeded 25×10^4 cells/cm²; in SV40 3T3 cells the volume decreased as the culture density exceeded 5×10^4 cells/cm² but the reduction may only account for a 7% loss of surface area. In 3T3 cells the volume decreased by 6% as the population density increased but the concomitant 3% decrease in surface area is too low to account for the reduction in Con A binding.

Fig. 4.18 Concanavalin A binding to SV40 3T3 cells with respect to cell population density.

The non-specific binding of Con A does not appear to alter significantly as the culture density increases from 4 to 15×10^4 cells/cm². However, the total binding decreases by 45% over the same range. In absolute terms the total binding decreases from 8.5 ± 0.8 to $4.7 \pm 0.6 \mu\text{g}/10^6$ cells ($p=0.001$ by linear regression analysis).

The columns are the mean of 4 experiments and the vertical bars are the standard errors of the means. The cross-hatched columns represent the non-specific binding.

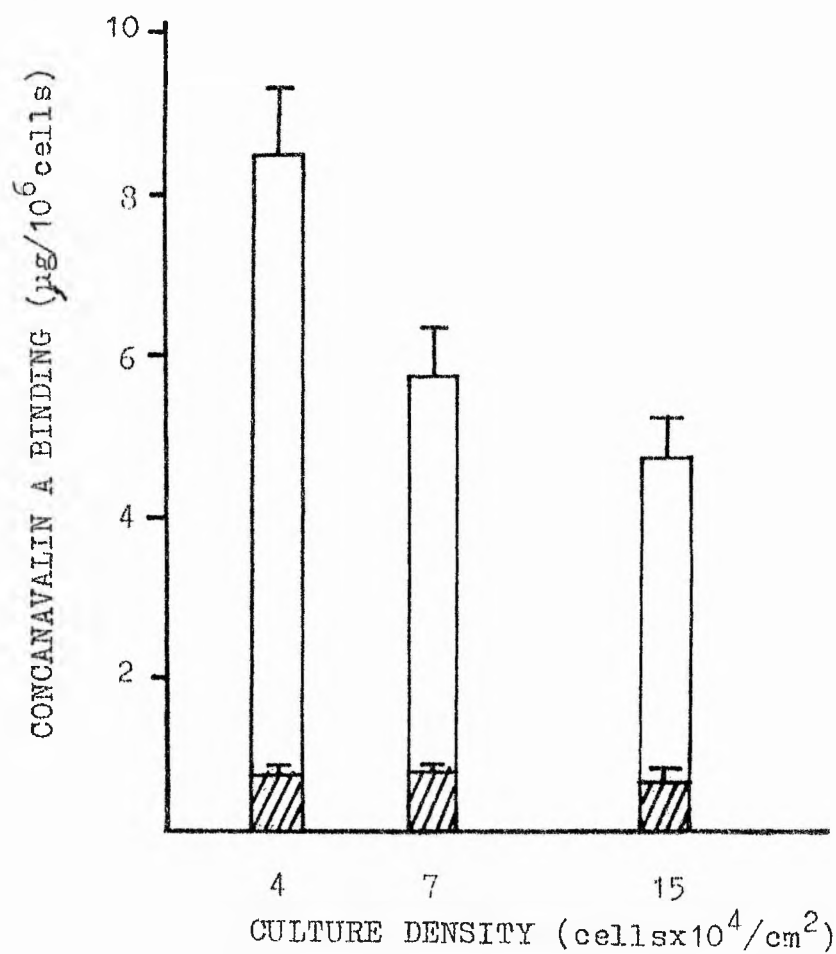


Fig. 4.18 The effect of cell population density on the binding of Concanavalin A to SV40 3T3 cells.

Fig. 4.19 Concanavalin A binding to 3T3 cells
with respect to cell population density.

As the culture density increases from 2 to 7 x 10^4 cells/cm² the total and non-specific binding both decrease by some 50% and when the cell population density reaches 11 x 10^4 cells/cm² both values are reduced by 65% (p=0.001 by linear regression analysis)

The columns are the mean of 4 experiments and the vertical bars are the standard errors of the means. The cross-hatched columns represent the non-specific binding.

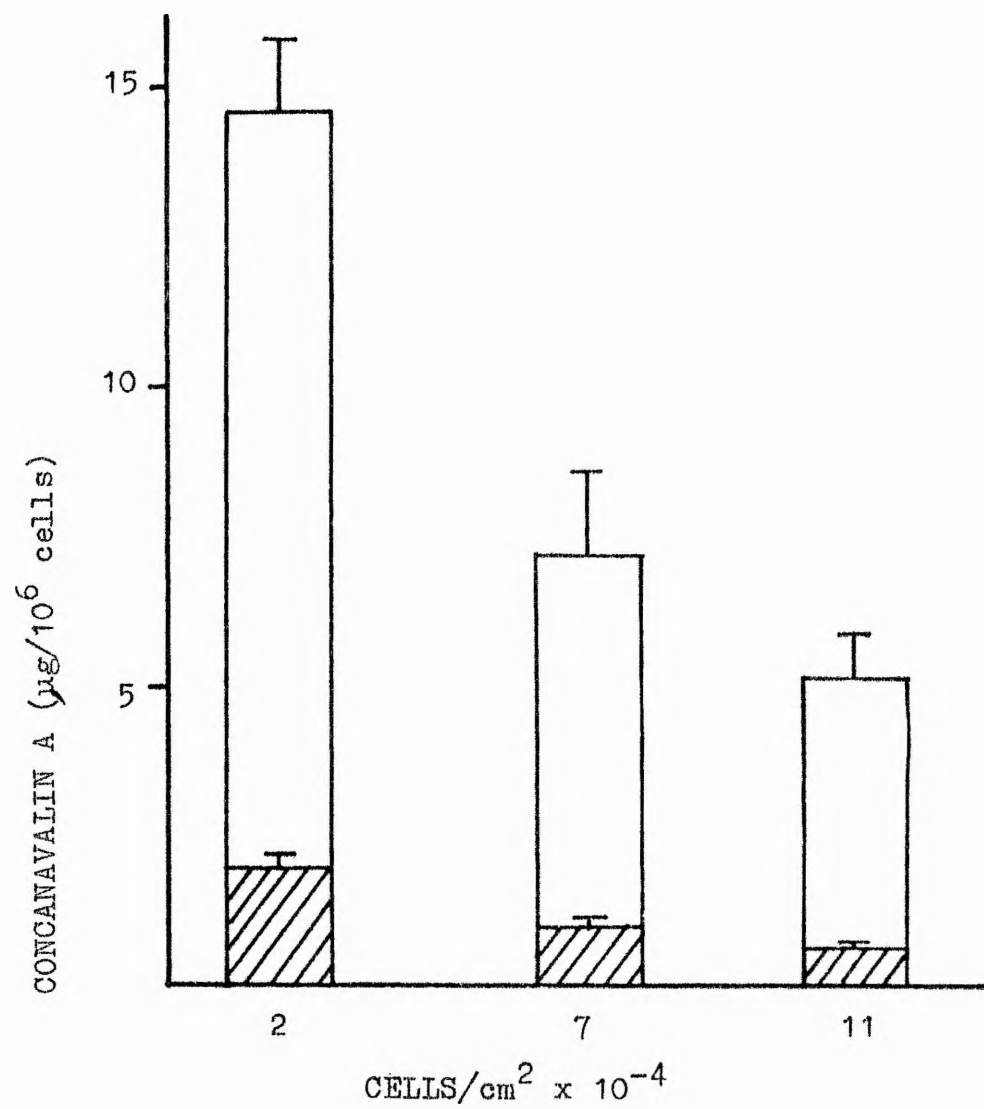


Fig. 4.19 Concanavalin A binding to 3T3 cells at different population densities.

In all the cultured cells examined, the cell volume did not appear to be related to the cell protein. In Py3T3 cells the protein content was reduced with density in a manner similar to that of the exo-ATPase; both were observed to decrease up to 15×10^4 cells/cm² and remain constant up to 30×10^4 cells/cm² (Fig. 4.4(a) and Fig. 4.3). However, cell volume, as measured in the Coulter counter (Fig. 4.4(b)) did not alter significantly until the density exceeded 25×10^4 cells/cm². In SV40 3T3 cells the cell protein did not alter significantly over the density range shown but the cell volume was significantly reduced as the density increased (Table 4.2). Similarly 3T3 cell volume did decrease but the cell protein showed no significant alteration with density. These observations are curious, since alterations in the cell volume might be expected to follow alterations in the cell protein if the latter were the main contributor to intracellular osmotic pressure.

Clearly, either the loss of protein does not affect the osmotic pressure of the cell or there must be some volume regulatory mechanism. The (Na+K)-ATPase decreases with density and the apparent Km for the enzyme is much lower than the reported cell ATP concentration (Graham 1972), so that all other factors being equal, the sodium pump is unlikely to be more able to regulate volume as the culture density increases. The possibility that the protein lost from the cells is from the exterior surface is dealt with in the Discussion section.

INTERNAL ENZYMES

In calf serum, the internal enzymes of SV40 3T3 and Py3T3

cells alter in a similar manner. In both cell lines the activity of acid phosphatase increased significantly as the culture density exceeded 5×10^4 cells/cm². The increment is 50% and 100% in Py3T3 cells and SV40 3T3 cells respectively (Tables 4.1 and 4.2). At the same culture density the rotenone insensitive NADH-ferricyanide reductase activity decreased in both cells by 35-40%. In neither cell line was the activity of monoamine oxidase affected by density. These observations are in contrast to the effects of culture density on 3T3 cells (Table 4.3). In these cells the activities of acid phosphatase, and monoamine oxidase increased by 3 and 2 fold respectively, while the rotenone insensitive NADH-ferricyanide reductase activity did not alter significantly.

In HeLa cells, (Table 4.4) culture density effected an increase in the rotenone insensitive NADH-ferricyanide reductase, while the other enzymes did not alter.

Cell surface and internal enzymes in foetal calf serum

Table 4.5 shows the effect of culture density on the activities of various enzymes of 3T3 and SV40 3T3 cells grown in foetal calf serum. This table is the mean of one experiment performed in triplicate and must, therefore, be interpreted with caution. However, it would appear that low density cultures of 3T3 cells in foetal calf serum contain activities of (Na+K)-ATPase and 5'-nucleotidase in the same range as those observed in newborn calf serum.

The effect of density on the intracellular enzymes of 3T3 cells is also dependent on whether the cultures were grown in

TABLE 4.1 The effect of cell population density on the activities of some of the 'intracellular' enzymes of Py3T3 cells.

The results are expressed in terms of cell protein and activities are compared at densities above and below 5×10^4 cells/cm². At this critical density there is a 1.5 fold decrease in the activity of rotenone insensitive ferricyanide reductase and a 1.5 fold increase in acid phosphatase activity ($p=0.01$ by student's T test). The activity of monoamine oxidase is not significantly affected by culture density.

The values given are the mean[±]SEM of 5-10 experiments.

TABLE 4.1 The effect of cell population density on the intracellular enzyme activities of Py3T3 cells.

<u>Enzyme</u>	Specific activity	
	<u>$\leq 5 \times 10^4$ cells/cm²</u>	<u>$> 5 \times 10^4$ cells/cm²</u>
Rotenone insensitive NADH ⁺ ferricyanide reductase	171 \pm 14	110 \pm 26
Monoamine oxidase ⁺	1.10 \pm 0.1	1.0 \pm 0.2
Acid phosphatase [*]	313 \pm 32	450 \pm 58

TABLE 4.2 The effect of culture density on several 'internal' enzymes of SV40 3T3 cells.

As the cell population density exceeds 5×10^4 cells/cm², rotenone insensitive NADH-ferricyanide reductase activity is reduced by 1.8 fold, acid phosphatase activity is increased by 1.9 fold ($p < 0.01$ in both cases), while monoamine oxidase activity and cell protein are not significantly affected. Cell volume decreases by 14% over the same density range ($p < 0.05$ by student T test).

The enzyme activity is expressed in terms of *nM/mg/Hr or [†]μM/mg/Hr and the values are the mean of 4-9 experiments [±]SEM.

TABLE 4.2 The effect of cell population density on
the intracellular enzyme activities of SV40 3T3 cells.

<u>Enzyme</u>	Specific activity	
	$\frac{< 5 \times 10^4 \text{ cells/cm}^2}{}$	$\frac{> 5 \times 10^4 \text{ cells/cm}^2}{}$
Rotenone insensitive NADH- ⁺ ferricyanide reductase	180 \pm 27	109 \pm 9
Monoamine oxidase ⁺	0.47 \pm 0.06	0.84 \pm 0.13
Acid phosphatase [*]	318 \pm 30	600 \pm 33
Protein(mg/10 ⁶ cells)	0.54 \pm 0.1	0.44 \pm 0.05
Volume(mls./10 ⁹ cells)	1.71 \pm 0.05	1.54 \pm 0.06

TABLE 4.3 The effects of culture density on several properties of 3T3 cells.

The activities of most of the enzymes were observed to increase as the culture density exceeded 5×10^4 cells/cm²; monoamine oxidase activity increased 3.7 fold, acid phosphatase activity increased 3.4 fold and cell volume decreased by 6% ($p < 0.02$ by student T test in all cases). There was no significant difference in cell protein or rotenone insensitive NADH-ferricyanide reductase as the density increased.

The activities are expressed as * nM/mg/Hr or ⁺ μM/mg/Hr, the values are the mean \pm SEM of 4-7 experiments.

TABLE 4.3 The effect of cell population density on
the intracellular enzymes of 3T3 cells.

<u>Enzyme</u>	Specific activity	
	<u><5x10⁴cells/cm²</u>	<u>>5x10⁴cells/cm²</u>
Rotenone insensitive NADH- ⁺ ferricyanide reductase	240 ± 25	271 ± 34
Monoamine oxidase ⁺	0.62 ± 0.07	1.44 ± 0.29
Acid phosphatase [*]	293 ± 40	992 ± 182
Cell protein(mg/10 ⁶ cells)	0.43 ± 0.04	0.32 ± 0.06
Cell volume(mls./10 ⁹ cells)	3.41 ± 0.07	3.20 ± 0.06

TABLE 4.4 The effect of cell population density on several of the properties of HeLa cells.

There is a 1.9 fold decrease in the activity of rotenone insensitive NADH-ferricyanide reductase as the culture density exceeds 5×10^4 cells/cm² (p=0.01 by student's T test) and none of the other properties alter significantly.

The enzyme activities are expressed as *nM/mg/Hr or [†]μM/mg/Hr; the values given are the mean of 4-7 experiments[†]SEM.

TABLE 4.4 The effect of cell population density
on the intracellular enzymes of HeLa cells.

<u>Enzyme</u>	Specific activity	
	<u><5x10⁴ cells/cm²</u>	<u>>5x10⁴ cells/cm²</u>
Rotenone insensitive NADH ⁺ ferricyanide reductase	160 \pm 20	84 \pm 8
Monoamine oxidase ⁺	0.75 \pm 0.3	0.70 \pm 0.1
Acid phosphatase [*]	41 \pm 3	35 \pm 6
Cell protein(mg/10 ⁶ cells)	0.32 \pm 0.05	0.30 \pm 0.05

TABLE 4.5 The effect of cell population density on various properties of 3T3 and SV40 3T3 cells grown in foetal calf serum.

In SV40 3T3 cells, the activities of (Na+K)-ATPase and acid phosphatase increase slightly with density, while the rotenone insensitive NADH-ferricyanide reductase activity decreases by some 30%. The effect of culture density in this experiment is different to that observed when the cells were grown in medium containing 10% newborn calf serum (Table 4.2).

When the cells are grown in medium containing 10% foetal calf serum, an increase in 3T3 cell culture density is associated with decreases in the activities of 5'-nucleotidase, (Na+K)-ATPase, rotenone insensitive NADH-ferricyanide reductase and monoamine oxidase, while the activity of acid phosphatase is observed to increase. These observations are again different from those when the cells are grown in medium containing 10% newborn calf serum.

The results are the mean of one experiment performed in triplicate, no statistics were performed on these results. ($A < 5 \times 10^4 \text{ cells/cm}^2$, $B > 5 \times 10^4 \text{ cells/cm}^2$)

TABLE 4.5 The effect of culture density on the activities of several of several of the enzymes of 3T3 and SV40 3T3 cells grown in foetal calf serum.

<u>Enzyme</u>	Specific activity			
	<u>SV40 3T3 cells</u>		<u>3T3 cells</u>	
	<u>A</u>	<u>B</u>	<u>A</u>	<u>B</u>
5'-nucleotidase [*]	190	160	190	46
(Na+K)-ATPase [*]	203	250	95	35
Rotenone insensitive NADH- ⁺ ferricyanide reductase	105	74	134	57
Monoamine oxidase ⁺	0.99	1.0	0.49	0.17
Acid phosphatase [*]	480	550	93	159

medium containing newborn or foetal calf serum. The only alteration which is similar in both cases is the increase in acid phosphatase activity with increasing population density but even in this case the activity of the enzyme at low culture densities and the magnitude of the increase are greater in calf serum. In newborn calf serum the activities of rotenone insensitive NADH-ferricyanide reductase and monoamine oxidase are reduced as the culture density increases while the activities of these enzymes are increased in calf serum under the same conditions.

The effect of cell population density on SV40 3T3 cells is also dependent on the type of serum present in the growth medium. Although the initial activities of 5'-nucleotidase and (Na+K)-ATPase appear to be independent of serum type, when the cells are grown in calf serum the activities decrease with increasing density and in foetal calf serum they do not alter markedly. Unlike the cell surface enzymes, the activities of acid phosphatase and monoamine oxidase at low culture densities are greater in foetal than in newborn calf serum and the activities increase with density in the latter while, in the former, they do not alter markedly. In contrast to the other 'intracellular enzymes', the activity of rotenone insensitive NADH-ferricyanide reductase was found to be higher in low density cultures grown in foetal calf serum than cultures grown in newborn calf serum but in both cases the activity was observed to decrease as the culture density increased.

The results presented here suggest that cell population

density exerts a profound influence on various Biochemical and Physiological properties of cultured cells and that the effects depend on the type of serum used to supplement the growth medium.

DISCUSSION

Intracellular enzymes

In both newborn and foetal calf serum, all the 3T3 cell lines examined exhibited an increase in acid phosphatase activity with increasing population density. Horvat and Acs (1973) have shown that the activity of lysosomal enzymes in 3T3 cells increases by 10 fold as the cells become confluent. When the cells were plated at day 0 and, each day for 25 days, measurements were made of DNA polymerase, aryl sulphatase and N-acetyl- β -D glucosamidase activities, the lysosomal activities were found to decrease by 3 fold over the first 3 days and, thereafter increase gradually to a maximum. In contrast, the DNA polymerase activity was low at day 1, increased to day 3 and then decreased with density. The increase in lysosomal enzymes is to be expected if there is a greater requirement for autosomal degradation of internal 'debris' as the average cell age increases. If the acid phosphatase activity measured in this report is representative of the lysosomal fraction, then its activity would be expected to increase for a similar reason and would occur whether the cells were grown in medium supplemented with newborn or foetal calf serum.

While the internal enzymes of SV40 3T3 cells altered in a similar manner with density in both newborn and foetal calf serum, the patterns of enzymic activities in 3T3 cells under these conditions were almost completely different. In the latter, monoamine oxidase activity increased with

density in calf serum and decreased with density in foetal calf serum while the rotenone insensitive NADH-ferricyanide reductase activity did not alter with density in calf serum and decreased in foetal calf.

The alterations in the effects of cell population density on the activities of these enzymes may be related to the ability of 3T3 cells in calf serum to attain higher confluent densities, but at the present time there is no good evidence for a causal relationship between these two events.

CELL VOLUME

Although cell surface area was not estimated in these experiments, routine measurements of cell volume were made. If the cells are, indeed, spherical after trypsinisation, and if their electrical resistance does not alter with density, then their Coulter volumes will be proportional to (surface area)^{3/2}. In Py3T3 cells there was no significant alteration in volume at densities below 25×10^4 cells/cm². However, as the culture density exceeded this value, the volume was reduced by some 21%, without any corresponding alteration in the activities of the cell surface enzymes. The volume of SV40 3T3 cells decreased by 10% as the activity of the (Na+K)-ATPase decreased by 3 fold. If the cells are perfect spheres, the 10% decrease in volume will represent a 7% loss of surface area. If the (Na+K)-ATPase activity is representative of sodium pumping, then the number of pump sites per unit surface area must drop sharply at this stage.

The volume of 3T3 cells in calf serum is reduced as the

culture density increases. This is, however, accompanied by an increase in the activities of the cell surface enzymes. Therefore, at densities greater than 5×10^4 cells/cm², the enzyme activity per unit surface area must increase or the membrane must be internalised. If the activity per unit surface area does increase with density, this occurrence does not permit the cells to go on dividing without limit and the onset of quiescence is not related, in all cases, to a decrease in the functional capacity of the plasma membrane. However, when the cells are grown in foetal calf serum, the decrease in the activities of the plasma membrane enzymes is associated with a reduction in the final density, but at the present time it is not possible to state whether these factors are causally related.

Cell Protein

Although the effect of density on the protein content of SV3T3 cells, 3T3 cells and HeLa cells is not statistically significant, there is a tendency for the cell protein to decrease as the culture density increases. In Py3T3 cell cultures there is a definite decrease in protein per cell with increasing culture density. This observation is in good agreement with those of Tsubio et al (1976) who observed a two fold decrease in protein per cell as L-cells attained high densities.

Although, in these experiments, the protein content of 3T3 cells did not appear to alter significantly with density Horvat and Acs (1973) observed a 40% decrease in 3T3 cell

protein as the culture density increased from 0.6 to 28×10^4 cells/cm² whereas Kimelberg and Mayhew (1976) reported a 10 fold decrease as the cell population density exceeded 0.2×10^4 cells/cm². The differences between these reports may be explained by a consideration of the constituents of the respective growth media. Mohammed (personal communication) has shown that 3T3 cells grown in foetal calf serum have a higher protein per cell at low culture densities than those grown in newborn calf serum, and that this value decreases to a greater extent in the former than in the latter. Kimelberg and Mayhew (1976) grew the cells in medium supplemented with 10% foetal calf serum while Horvat and Acs (1973) grew their cells in medium containing 5% foetal and 5% newborn calf serum and in the present study the cells were grown in 10% newborn calf serum.

Although cell protein is the main intracellular osmotic component, the cell volume does not appear to be directly related to this factor. There may be several ways of explaining this observation. The two most obvious are 1) the cell is able to compensate for the loss of protein by increasing the sodium pump activity. This appears unlikely, since the (Na+K)-ATPase activity actually decreases with density, or 2) the protein decrease is due to the loss of material from the extracellular surface. Pasternak (1976) has observed that trypsinisation of cells results in a loss of cell protein. When the cells are then incubated in serum containing medium they bind serum proteins to their surface. During the process of binding surface

proteins the protein per cell may increase as much as two fold. In these experiments, the protein content of SV40 3T3 cells altered markedly with the serum concentration of the growth medium while the observed Coulter volumes were not significantly affected. If the protein which was lost from the cells as the density increased, was lost from the exterior of the cell surface, then there would be no loss of osmolarity and no need for a compensatory mechanism.

Cell surface enzymes

In HeLa cells grown in 10% calf serum the cell surface enzymes were affected by culture density in a manner very similar to that of transformed 3T3 cells. However, the alteration in (Na+K)-ATPase is not directly reflected by the alterations in the specific ouabain binding. The latter appeared to occur to a different extent and at a different density from the former. As already mentioned in the results section these parameters need not be directly related. However, when the rate of hydrolysis of ATP by the specific ouabain binding sites is predicted by assuming that each site completes 30 pumping cycles per second, the values obtained for cultures at densities less than 2×10^4 cells/cm² and greater than 5×10^4 cells/cm² are in good agreement with the rate of hydrolysis of ATP by the (Na+K)-ATPase. At densities less than 2×10^4 cells/cm² the rate of hydrolysis of ATP by the sodium pump is $6.0 \pm 0.7 \times 10^7$ molecules/cell/sec. while the rate of hydrolysis by the (Na+K)-ATPase is $6.0 \pm 0.5 \times 10^7$ molecules/cell/sec. At densities greater than 5×10^4 cells/cm²

the former value is reduced to $2.8 \pm 0.3 \times 10^7$ molecules/cell/sec. while the latter is $1.8 \pm 0.4 \times 10^7$ molecules/cell/sec. The fact that the latter value is less than the former is probably due to an inactivation of the enzyme on homogenization or to the formation of closed vesicles which renders the active site of the enzyme cryptic. At very low population densities, both the predicted and experimental rates of hydrolysis agree but as the density exceeds 1×10^4 cells/cm², the ouabain binding decreases gradually while the (Na+K)-ATPase activity does not decrease until the density exceeds 5×10^4 cells/cm². Although these results were obtained from different experiments they suggest that the pump sites may be removed from the cell surface prior to loss of the enzyme activity or that they become cryptic and that the activity is released on hydrolysis.

Grinstein and Erlj (1974) demonstrated an immediate increase in ³H ouabain binding to frog sartorius on treatment with insulin; this effect was not abolished by cycloheximide and occurred when the muscle was preincubated with ouabain suggesting that there had been an uncovering of pump sites. Wiesmenn et al (1976) using insulin to stimulate Na⁺ efflux from toad bladder, showed that there were 2 phases of stimulation, acute and chronic. The former which was unaffected by actinomycin D was claimed to be due to an uncovering of cryptic sites. Quastel et al (1970, 1972) and Wright et al (1973) have suggested that the immediate 2-3 fold increase in ³ouabain/³H binding and ouabain sensitive influx into cultured lymphocytes, which is stimulated by phytohemagglutination, is caused by a conformational change in the plasma membrane which

exposes previously cryptic sites. However, Averdunk and Luaf (1975) have since concluded that the response is due to a conformational change in the sodium pump which alters its kinetics and affinity for ouabain. Furthermore, if there exists, in low density cultures of HeLa cells, an intracellular pool of sodium pump sites similar to the acetyl choline receptors of cultured chick skeletal muscle (Hartzell and Fambrough 1973), and a component of the pool is readily incorporated into the plasma membrane, there would be no requirement for cryptic sites at the cell surface.

In the transformed 3T3 cell lines, the activities of the cell surface enzymes were observed to alter differently as the culture density increased. The (Na+K)-ATPase decreased sharply as the culture density exceeded 5×10^4 cells/cm², while the activity of the 5'-nucleotidase decreases gradually. However, the activity of the 5'-nucleotidase levelled off at higher densities, so that the overall extent of the reduction is similar for both enzymes.

Aiton (1976) suggested that there was a factor in calf serum which effected an increase in the number of sodium pumps in HeLa cells. The factor appeared to be removed from the medium during the growth of the cells. Experiments reported here have shown that, at least in SV40 3T3 cells, the decrease in (Na+K)-ATPase is not simply due to the removal of stimulatory material from the growth medium. This follows from the observation that the activity of the (Na+K)-ATPase did not increase on preincubation of the cultures with fresh medium containing 10% calf serum. In 0 and 20% serum the level of this activity altered but the alterations were

similar to the changes observed in all of the other enzymes measured. The activity of the (Na+K)-ATPase was only specifically increased by fresh medium containing 10% serum, after trypsin treatment. These results are, however, not inconsistent with those of Aiton since he could have monitored the incorporation of sodium pumps into the membrane from an intracellular pool. Alternatively, in HeLa cells, prior trypsinisation might not be necessary for the increase to occur.

The effect of culture density on the activity of the cell surface enzymes of 3T3 cells in calf serum is distinct from the effect on transformed 3T3 cells and HeLa cells. In the former, the activities of the cell surface enzymes increase with culture density, while in the latter they decrease. These observations do not agree with those of Lelievre et al (1973) who showed that in contact inhibited plasmocytoma cells, the activities of the (Na+K)-ATPase and 5'-nucleotidase decreased by 4 and 3 fold respectively, as the cell density increased from 4×10^4 cells/cm² to 8×10^4 cells/cm². In non-contact inhibited cells the activities of these enzymes did not alter over the range $5-20 \times 10^4$ cells/cm². It is interesting to note that the density at which these authors report a decrease in the activity of the cell surface enzyme is approximately the same as that reported here for transformed 3T3 cells. On the other hand the lowest density of their non-contact inhibited cells was 5×10^4 cells/cm², so that any increase as the cells exceeded 5×10^4 cells/cm² would not have been observed in their investigation. Elligsen et al (1976) report similar observations to those of Lelievre et al.

The former, using normal and virus transformed 3T3 cells, showed that there was a decrease in the activity of (Na+K)-ATPase as the density of 3T3 cells increased above 5×10^4 cells/cm², while the activity in transformed cells was independent of density.

The alterations in the activities of the (Na+K)-ATPase in normal and virus transformed cells reported here are such that at low cell densities, the activity is higher in transformed cells while at higher cell densities, the activity is greater in 3T3 cells. The literature on comparisons of the activity of the (Na+K)-ATPase in virus transformed and normal cells tends to be contradictory. The confusion is probably aggravated by the fact that authors often omit to state the culture density at which their experiments were performed.

On a close examination of the literature, however, a pattern emerges. Elligsen et al (1976), Kimelberg and Mayhew (1975) and Kasarov and Freidman (1974) observed that the activity of the (Na+K)-ATPase in transformed cells was greater than in normal cells. However, Graham (1972), Yoshikawa-Fudaka and Najima (1972) and Perdue et al (1971) report that transformation leads to a decrease in the activity of that enzyme. Where an increase has been reported in normal cells, the cells have been grown in calf serum, and where a decrease has been reported, the cells have been grown in foetal calf serum. The serum dependence of the alterations in the levels of (Na+K)-ATPase has been observed in this laboratory (Mohammed personal communication) and preliminary experiments reported here confirm this

observation. In normal 3T3 cells grown in foetal calf serum, the activities of the cell surface enzymes decreased with density, while in transformed cells, under the same conditions, the activities did not alter markedly.

If the results reported here are valid then much of the confusion in the literature may be resolved. For example, the experiments of Elligsen (1976) which show a decrease in the activity of the (Na+K)-ATPase in normal cells as the density increased, while the activity in transformed cells was not affected by culture density, were performed using foetal calf serum. His results then agree with those reported here.

In addition to serum effects the activity may also be related to the number of passages which the cells have undergone. Tupper (1977) has shown that in 3T3 cells after 130 passages in calf serum, the final density increased from 5×10^4 to 20×10^4 cells/cm² and the ouabain sensitive potassium flux increased by approximately 5 fold. In these experiments 3T3 cells grown in foetal calf serum almost attain the higher densities reported by Tupper (1977). (Mohammed, personal communication, has obtained densities up to 20×10^4 cells/cm².) However, when the cells are passaged into foetal calf serum, the final density is reduced to 9×10^4 cells/cm² or less, while the (Na+K)-ATPase activity is reduced from 2-10 fold depending on the density at which the comparison is made. It seems not unlikely that the transfer of cells from newborn to foetal calf serum might result in a regression of the cells to a state comparable to that

observed after a few passages in calf serum. If this were true then the increase induced after prolonged culture in calf serum might be either due to a selection of a cell population which would attain higher densities and which had a higher activity of $(\text{Na}+\text{K})$ -ATPase activity, or the prolonged growth in calf serum alters the enzymic characteristics of the cells. Clearly the former does not hold since reversal of the effect occurs so readily in foetal calf serum. It seems more likely that the effect is produced by treatment with calf serum. There are precedents for this sort of effect. Gyévia and Fazekos (1971) observed a similar phenomenon with HeLa cells in culture. When HeLa cells, obtained from a woman of group O blood, were grown for 10 days in media containing 10% group O, B or A serum instead of calf serum, there were no alterations in the activities of acid phosphatase or non specific esterase. However, after 55-60 days in these media the activities of these enzymes were observed to change. Cells grown in group O serum had reduced levels of both enzymes, those grown in group B serum contained levels of both enzymes significantly increased over controls while cells cultured in group A blood contained increased activity of the acid phosphatase only.

The effect of cell density on the levels of cell surface enzymes is not restricted to mammalian cells. Dictostelium discoideum cells in the stationary phase of growth contain an activity of 5'-nucleotidase which is one tenth that observed in the exponential phase. Nor are alterations of this sort restricted to systems in vitro. Weimar and Haraguchi (1967) have shown that wound repair in the cornea is associated with

"a remarkable series of changes in enzymology, physiology and morphology of connective tissue cells in the wound area within a few hours after injury". Normal epithelial, endothelial and stromal cells do not stain positive for 5'-nucleotidase. However, as the inflammatory response to a lesion increased, the development of 5'-nucleotidase increased. When the inflammatory response was poor, the activity was associated with a region immediately round the wound but the activity spread as the area of the response increased. As the tissue healed, 5'-nucleotidase activity decreased until, after 96-168 hours, the activity was again restricted to the immediate vicinity of the wound.

In experiments reported here, the activity of the cell surface enzymes was, depending on the cell type, related to both the cell density and to the serum content of the medium. However, further experiments would be required to conclusively prove the involvement of serum. It would have been instructive, during these experiments, to relate the activity of the cell surface enzymes to the cell surface area, but such an investigation was already being performed in this laboratory. Clearly, however, if the activities of both 5'-nucleotidase and (Na+K)-ATPase are located in the cell membrane and, as observed, the decrease in the activity of the (Na+K)-ATPase occurs sharply while the activity of the 5'-nucleotidase occurs gradually, then at least one of these activities is not a simple function of the cell surface area.

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